

Immunological Properties of Teichoic Acids

K. W. KNOX AND A. J. WICKEN

Institute of Dental Research, United Dental Hospital, Sydney, New South Wales 2010, and School of Microbiology, University of New South Wales, Kensington, New South Wales 2033, Australia

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INTRODUCTION

Teichoic acids have been known since 1959 to be components of the outer layers of probably all gram-positive bacteria; they apparently are not synthesized by any gram-negative bacteria. Conventionally, teichoic acids are regarded as

either cell wall or cell membrane components, the latter being the operational fractions obtained from disrupted bacteria from which teichoic acids may be extracted. Such distinctions may imply a locational significance which is not

absolute, particularly in relation to the in situ serological activity of these polymers. For this reason, it is perhaps more meaningful to consider teichoic acids on the basis of their covalent linkage either to peptidoglycan (wall teichoic acids) or a membrane-associated lipid (membrane teichoic acids); the question of cyto-logical location can then be considered as a separate issue. Although the occurrence of wall teichoic acids is variable between different genera, membrane teichoic acids appear to be virtually ubiquitous in gram-positive bacteria. The detailed structures, function, and biosynthesis of these polymers have been the subjects of extensive investigations since the time of their discovery and have been equally extensively reviewed (10, 13, 14, 28, 29).

Membrane-associated teichoic acids are characterized by their uniformity of structure. These polymers possess a linear backbone of poly (glycerol phosphate) in which linkage is through phosphodiester groups involving positions one and three of adjacent glycerol residues (Fig. 1). Structural variation in this group of teichoic acids appears to be confined to the nature and extent of glycosyl substitution of the secondary hydroxyl groups (position two) of the glycerol units. D-Alanine ester residues are usually also found as substituents of either glycerol or glycosyl hydroxyl groups. It now appears likely, as is discussed later in this review, that most, if not all, membrane teichoic acids are linked covalently to membrane glycolipid, and the term lipoteichoic acid has been given to such complexes (295).

Peptidoglycan- or wall-associated teichoic acids, on the other hand, are remarkable for their structural diversity, and it is difficult to define what is and what is not a teichoic acid. Baddiley (29) suggests that the term might encompass all "polymers that possess phosphodiester groups, polyols, and/or sugar residues, and usually, but not always, D-alanine ester residues." The simplest and, for the purpose of this review, classical wall teichoic acids are either glycerol teichoic acids similar to the membrane-associated polymers (Fig. 1) or ribitol teichoic acids in which ribitol replaces glycerol as the backbone polyol unit (Fig. 2). These polymers contain only a restricted array of sugar substituents (Table 1).

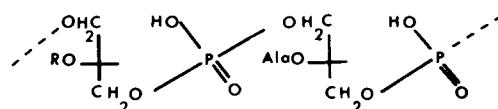


FIG. 1. A glycerol teichoic acid. R, H or glycosyl; ala, D-alanyl.

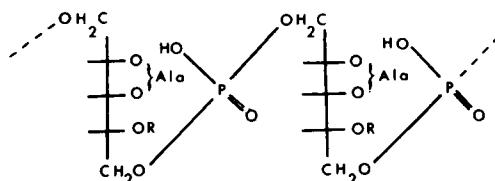


FIG. 2. A ribitol teichoic acid. R, H or glycosyl; ala, D-alanyl.

Although these structures describe the majority of teichoic acids that have been studied, there are variations of structure among wall teichoic acids, which are of considerable significance to the overall shape or conformation, and, in turn, the comparative serological specificity of these molecules. Glycerol teichoic acids with phosphodiester linkages between positions two and three, rather than one and three, of the glycerol residues have been reported from the walls of *Bacillus stearothermophilus* (290) and *Actinomyces antibioticus* (203). Other variations of structure that have been encountered mainly center around inclusion of sugar residues in the backbone of the polymer, and a wide range of polymers have been isolated and characterized (Fig. 3). The number of sugar residues (x in Fig. 3) may vary from a single hexose unit as in *Bacillus licheniformis* (96), through disaccharides as in *Lactobacillus plantarum* C 106 (23), to oligosaccharides of several different sugars as occur in the type specific capsular substances of some pneumococci (231, 236, 250). Teichoic acids of this type (Fig. 3) may also have side-chain substitution of the polyol residue as is found, for example, in the wall teichoic acids of *Bacillus coagulans* (88, 89) and pneumococci (42). The latter pneumococcal C substance, has been partially characterized as having a poly (diamino-sugar-ribitol phosphate) backbone variously substituted with choline phosphate.

Polymers in which N-acetylglucosamine-1-phosphate is attached through phosphodiester linkage to glycerol phosphate (Fig. 4) have been isolated from the walls of "Staphylococcus lactis" I3 (= *Micrococcus lactis*; 32, 33) and *Micrococcus* sp. 24 (18, 26). Related polysaccharide polymers, where the monomer units are sugar-1-phosphates, have also been isolated from the walls of some *Micrococcus* species (18). While most wall teichoic acids are substituted with D-alanyl esters, these components may be replaced in rare instances by succinate (*Actinomyces streptomycini*, 201) or acetate (*Actinomyces violaceus*, 202).

It is generally recognized, from the results of several studies, that teichoic acids, free of

TABLE 1. Examples of carbohydrate components of teichoic acids

Location	Polyol	Carbohydrate(s) ^a	Occurrence	References
Membrane	Glycerol	Glc Glc- α -1 → 2-Glc Glc- β -1 → 6-Glc Gal, Gal- α -1 → 2-Glc	<i>L. helveticus</i> NCIB 7220 <i>S. faecalis</i> 39 <i>S. aureus</i> H <i>L. fermenti</i> NCTC 6991	63, 254 291 223 295 ^b
Wall	Glycerol	Glc	<i>B. subtilis</i> Marburg <i>L. buchneri</i> NCIB 8007 <i>S. epidermidis</i> T1 <i>S. epidermidis</i> (<i>albus</i>) NCTC 7944	49 21 65 81
		GalNAc	<i>B. subtilis</i> W-23 <i>L. plantarum</i> (<i>arabinosus</i>) 17-5 <i>S. aureus</i> Copenhagen <i>S. aureus</i> phage type 187	189 14 280 152
	Ribitol	Glc		
		GlcNAc GalNAc		

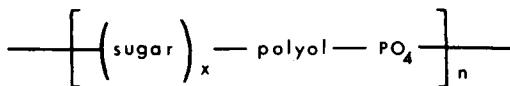
^a Glc, D-glucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine.^b A. J. Wicken and K. W. Knox, unpublished data.

FIG. 3. Repeating unit of a teichoic acid with one or more sugar components in the "backbone." Polyol is either glycerol or ribitol.

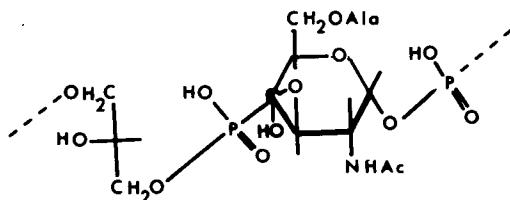


FIG. 4. Teichoic acid containing N-acetylglucosamine-1-phosphate as a "backbone" component. Ala, D-alanyl; Ac, acetyl.

association with other wall or membrane components, do not induce antibody formation, whereas they will react with antibody formed against the appropriate bacterial cell or cell fraction. This distinction is well known among other categories of serologically reactive substances and led to the use of the terms "antigen" (inducing antibodies) and "hapten" (serologically reactive). Currently, the term "immunogenic" is preferred for indicating the capability of a substance to induce an immunological response whereas "antigenic" relates more to its reactivity and specificity in a reaction.

The use of the term "group antigen" preceded this newer distinction, but would still seem an appropriate definition of a bacterial component; the definition of a group antigen has usually preceded its identification, and in many instances the isolated antigen does not induce antibody formation. Similarly, the use of the term "antigen-antibody reaction" would seem

valid in referring to the various detection systems discussed in this review, in which the reaction observed depends on a particular component of an immunogenic complex and in which the isolated component itself is not necessarily immunogenic. For example, and as is discussed later, the lipoteichoic acid-protein complex from the membrane of lactobacilli is immunogenic; specificity depends on the teichoic acid component but this component alone, although reacting with antibodies in certain systems, will not induce antibody formation (160).

LOCATION OF TEICHOIC ACIDS

Isolation and purification of bacterial cell walls has generally been considered as the first step in the extraction of wall teichoic acids in order to avoid contamination with membrane teichoic acid. The assumption that cell walls can be prepared free of membrane material is, however, not valid in every case. Cell walls of gram-positive organisms, prepared conventionally by mechanical disruption, treatment with deoxyribonuclease and ribonuclease, and repeated washing with buffers and water, frequently contain small quantities of lipid (85, 88, 242). Recent studies (194) on cell walls similarly isolated from strains of *Staphylococcus aureus* have shown that the lipid content is due to residual membrane which may be either physically joined to the wall or trapped by the collapse of the rigid wall during disruption of the cells. Membrane teichoic acid was associated with these membrane components, and small amounts of teichoic acid and phospholipid still persisted as wall contaminants even after additional purification procedures, among which heating, proteolysis with trypsin,

and treatment with detergents were the most effective. Membrane teichoic acid was also found to be still associated with cell walls of *L. plantarum* after treatment of the latter with trypsin (164). Caution must therefore be exercised in interpreting serological activities of extracts of cell walls. For a general and practical account of the preparation, purification, and analysis of bacterial cell walls we recommend the recent review by Work (305).

Teichoic Acid-Peptidoglycan Complexes

Evidence for a covalent linkage between teichoic acid and peptidoglycan. It has long been evident that the conditions required for the complete extraction of teichoic acids from cell walls involve covalent bond breakage if the product obtained is to be free of other wall material. Teichoic acid-peptidoglycan complexes from enzymic digests of cell walls were devoid of detectable phosphomonoester groups, suggesting that the terminal phosphomonoester group of the teichoic acid was involved in a linkage with a component of peptidoglycan (275). Muramic acid phosphate was identified in 1958 (5), and there is now evidence that the phosphate grouping of this component provides the cross-link between the terminal polyol unit of teichoic acid and peptidoglycan (46, 105).

A relatively acid-labile phosphoramidate bond between the terminal phosphate group of teichoic acid and a hexosamine amino group of the peptidoglycan has also been proposed for organisms whose wall teichoic acid is fairly readily extracted under acid conditions (121). Although the existence of such a linkage still has to be demonstrated, the possibility of there being more than one type of linkage of teichoic acid to peptidoglycan may be indicated by the observations of rapid release of teichoic acid from some organisms under alkaline conditions (130), whereas less than 10% of the wall teichoic acid of *B. stearothermophilus* was solubilized in 48 h by the same procedure (105). The degree of extractability of teichoic acid from *Bacillus subtilis* var. *niger* with trichloroacetic acid (79) has been noted to vary with the growth conditions employed. A phosphodiesterase capable of depolymerizing teichoic acid has been obtained from *B. subtilis* (302), and the possibility of such an enzyme occurring in some bacteria and causing partial degradation of teichoic acids during the preparation of cell walls or extraction of teichoic acid should not be ignored.

In view of the structural diversity of wall teichoic acids and possible different modes of linkage to other cell wall polymers, it is not surprising that a variety of procedures have

been developed for their isolation in polymeric form. No single procedure is likely to extract all teichoic acids without degradation, and for serological and other studies, teichoic acid preparations extracted by different procedures should be compared.

Extraction of peptidoglycan-free teichoic acid. Complete extraction of teichoic acid from walls free of association with peptidoglycan occurs only slowly at 4°C in dilute trichloroacetic acid solutions (5 to 10%), but this is probably the most widely used, and indeed classical, procedure (27). There has been some controversy as to whether these acidic conditions cause degradation of wall teichoic acid through hydrolysis of inter-unit phosphodiester bonds (95). A careful re-examination of the trichloroacetic acid-extracted teichoic acid from the walls of *S. aureus*, *B. subtilis*, and *Lactobacillus arabinosus* (122) suggested that the rate of such a hydrolysis is low under the conditions employed for extraction, and little degradation occurs. However the product obtained with trichloroacetic acid may not be representative of all of the teichoic acid(s) in the wall. With *B. subtilis* W23, which contains two ribitol teichoic acids, trichloroacetic acid-extraction removes most of the glucosyl-substituted polymer, but only small amounts of the unsubstituted polymer (49). Similarly, with *Bacillus subtilis* Marburg, where the wall contains glycerol teichoic acids, the extract contained mostly the glucosylated polymer (49). Studies on trichloroacetic acid extracts from *B. licheniformis* (135), *B. subtilis* (310), and *B. stearothermophilus* (105) also indicated that the extracted teichoic acid was not representative of the wall polymers.

A serological examination of extracts of *L. plantarum* NCIB 7220 provided evidence for ribitol teichoic acid molecules differing in glucosidic substitution over at least a fourfold range (164). The molar ratio of glucose to phosphorus in the extract was higher than in unextracted walls, suggesting that nonglucosylated ribitol teichoic acid was present in the wall, but was not recovered in the extract. The apparent absence of nonglucosylated teichoic acid in trichloroacetic acid extracts may reflect differences in the ease of extraction rather than degradation; no difficulty, for instance, was experienced in extracting the nonglucosylated ribitol teichoic acid from *L. plantarum* ATCC 10241/R1 where it was the only teichoic acid component (73, 164).

Although exposure to trichloroacetic acid in the cold for relatively short periods (16 h) probably avoids extensive degradation of tei-

choic acids, yields are often rather low (20 to 30%). Complete extraction usually requires prolonged exposure or higher temperatures, conditions which can bring about hydrolysis of glycosidic as well as phosphodiester bonds; treatment of *S. aureus* wall teichoic acid with trichloroacetic acid for 15 min at 90°C resulted in almost complete loss of serological activity (110).

To avoid the possibility of hydrolysis of glycosidic linkages, wall preparations have been extracted under various alkaline conditions, usually 0.1 N NaOH either at room temperature or 35°C (20, 25, 137). Alanine ester linkages would be hydrolysed, but, depending on the type of teichoic acid structure, interunit phosphodiester bonds may also be broken (20). Generally, the peptidoglycan component is not solubilized, although *S. aureus* provides an exception because of the lability of the glycyl linkages in the cross-bridges (19). In specific cases the method may prove useful for the differential extraction of cell wall components, for it has been shown that alkali treatment of *B. licheniformis* wall solubilizes the teichoic acid component but not teichuronic acid (137), a polymer of glucuronic acid and *N*-acetylgalactosamine (138, 145). The term "teichuronic acid," although originally applied to this polymer is now used more generally to refer to gram-positive bacterial cell wall polysaccharides containing uronic acids.

A method which has the potential for being generally applicable to the extraction of serologically active teichoic acids is the treatment of walls with phenylhydrazine (12, 111) or (to avoid the formation of tar) *N,N*-dimethylhydrazine (7). The method, which probably involves free radicals, gives high yields of both polysaccharides and teichoic acids, completely removing teichoic acid from *S. aureus* wall without detectable loss of serological activity (111).

Older methods used for the extraction of antigenic material from bacteria such as formamide at 150 to 160°C (92) or HCl at pH 2 and 100°C (170) have found little application in the isolation of teichoic acids, and in view of the extreme conditions involved and consequent risk of degradation are best avoided.

Extraction of peptidoglycan-associated teichoic acid. To obtain soluble teichoic acid-peptidoglycan complexes requires hydrolysis of linkages within the peptidoglycan component, and a wide variety of suitable enzymes has been described; these include carbohydrases and peptidases of plant, animal, and bacterial origin as well as endogenous wall autolysins (94, 276). Fractionation of the hydrolysates by ion ex-

change chromatography or gel filtration yields teichoic acid-peptidoglycan complexes with higher molecular weights than free teichoic acids, and therefore they are more suitable for serological reactions (95, 105, 106, 168, 310).

A novel and potentially useful approach to the purification of teichoic acids in enzymatic digests or other extracts depends on the affinity of the plant lectin concanavalin A for the α -anomers of *D*-glucose, *N*-acetyl-*D*-glucosamine and *N*-acetyl-*D*-galactosamine (74). The method employs a column of cyanogen bromide-activated agarose gel which will bind concanavalin A. When an autolysate of *B. subtilis* 168 was passed through the column, only teichoic acid-containing fractions were retained, and these could be eluted with methyl- α -*D*-glucopyranoside. The resultant product was less polydisperse than teichoic acid obtained by acid extraction. (Con A-Sepharose is now available from Pharmacia Ltd., Uppsala, Sweden.)

Teichoic Acids as Membrane Components

Evidence for location. In contrast to the variable occurrence of peptidoglycan-associated wall teichoic acid, most gram-positive bacteria contain a glycerol teichoic acid that can be isolated from the intracellular fraction of disrupted cells. Although initially referred to simply as "intracellular teichoic acid" (59), it was found that the teichoic acid was present in the particulate ribosome-membrane fraction (59, 291). Subsequent studies based on an examination of products resulting from protoplast formation led to the introduction of the term "membrane teichoic acid." It was found from chemical analysis that the formation of protoplasts from *Streptococcus faecalis* and *Bacillus megaterium* was associated with the release of the major portion of the teichoic acid into the soluble fraction, although some remained associated with the cell membrane (123). Similar findings were obtained with several strains of streptococci by using serological techniques to identify teichoic acid (258, 270). A detailed investigation on *S. faecalis* ATCC 9790 (*S. faecium*) (263) showed that the teichoic acid was associated with the membrane from which location it could readily be removed by washing with water or salt solutions. All of these investigations strongly suggested that membrane teichoic acid was located in or on the external surface of the protoplast membrane, although the nature of the attachment remained obscure. It now seems probable that membrane association depends on a covalent linkage between teichoic acid and membrane glycolipid (see below).

More direct evidence for membrane association has been provided by the use of ferritin-labeled antibodies, and Fig. 5 shows the result of reacting protoplasts of *Lactobacillus fermenti* NCTC 6991 successively with antibodies prepared against the membrane teichoic acid and ferritin conjugated to antirabbit gamma globulin (283). The antiteichoic acid antiserum used was prepared by injection of the lipoteichoic acid-protein complex into rabbits (see Immunogenicity of Teichoic Acid) and was specific for the teichoic acid moiety of the complex, and no serological activity towards either the protein or lipid portions of the complex was detected. Teichoic acid from which both protein and lipid had been removed completely absorbed antibodies (294) from the antiserum. (This restriction of antibody specificity to the teichoic acid portion of lipoteichoic acid-protein complexes appears to be a common one and is discussed later in this review.)

Extraction of lipid-free teichoic acid. Membrane teichoic acids were originally obtained by trichloroacetic acid extraction of the 100,000 g membrane-ribosome fraction from disrupted organisms after removal of cell walls (59). Crude extracts were heavily contaminated with poly-

nucleotides, polysaccharide, and varying amounts of wall teichoic acid; the latter contaminant may have represented fragmentation of the wall during the disruption procedure or partially synthesized and still membrane-associated wall teichoic acid. Trichloroacetic acid-extraction of whole organisms, as might be expected, increases the amount of contamination by wall polymers. Fractional precipitation with alcohol or acetone followed by chromatography on columns of ion-exchange celluloses or low porosity gels has been used to obtain membrane teichoic acids with a high degree of purity and free of association with other cellular components (59, 291). Such preparations had average chain lengths of 17 to 22 glycerophosphate units and molecular weights in the range 3,000 to 12,000, depending on the degree of sugar substitution. Gel chromatography of trichloroacetic acid-extracted material gave a small fraction of the teichoic acid apparently bound to polynucleotide, and such "complexes" were excluded from the low-porosity gels (Sephadex G75) available at that time (291). In the light of more recent studies (see below) it appears likely that the higher-molecular-weight material represented partially degraded lipotei-

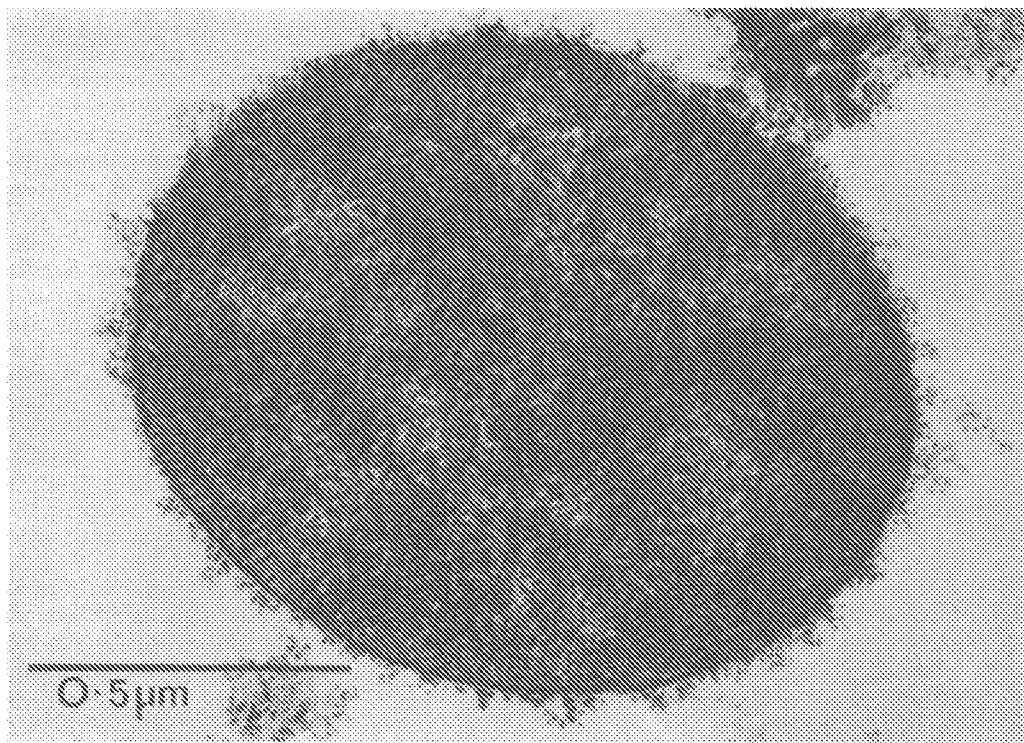


FIG. 5. Protoplast of *L. fermenti* treated with antiserum to the lipoteichoic acid and then reacted with ferritin conjugated to goat antirabbit gamma globulin. [from *Journal of Ultrastructure Research* (283).]

choic acid and that the association with polynucleotide was more apparent than real. It is, however, of some practical interest to note that preparations of bacterial ribosomes (291) and deoxyribonucleic acid (DNA) (311) are invariably contaminated with membrane teichoic acid, presumably as a result of cellular disruption. Teichoic acid has also been reported as both a contaminant of transfer ribonucleic acid (tRNA) of *B. subtilis* and an inhibitor of accepting activity for tRNA^{Phe} and tRNA^{Tyr} (308).

Older methods for the extraction of serologically reactive teichoic acids from bacteria, including formamide at 150 to 160 C (92), dilute acid (170), and mild alkaline conditions (76) are likely to yield degraded products. For example: (i) deacylation of lipoteichoic acids would be expected, (ii) there is the previously mentioned possibility of interunit phosphodiester bonds being hydrolyzed by acid, and (iii) glycosidic bonds may be particularly susceptible to acid hydrolysis through the influence of neighboring phosphate groups (291). Differences that probably relate to the lability of glycosidic bonds have been found when comparing the serological reactions of Lancefield acid extracts and formamide extracts (131) and also of Lancefield acid and trichloroacetic acid extracts (292) from group D streptococci with homologous antiserum where specificity depends on the 2-O- α -D-glucosyl-D-glucose (kojibiose) substituent (43).

Extraction of lipoteichoic acids. High-molecular-weight preparations of teichoic acid were obtained from *B. licheniformis* by cold phenol extraction (44), and it was suggested that prolonged exposure to the acid conditions of trichloroacetic acid-extraction might cause the hydrolysis of phosphodiester linkages. The possibility that the less drastic conditions of extraction afforded by cold phenol might provide a means of isolating "native" membrane teichoic acid still associated with other cellular components prompted us to compare the properties of phenol- and trichloroacetic acid-extracted preparations of membrane teichoic acid from *L. fermenti* NCTC 6991 (295).

The cell contents from disrupted *L. fermenti* were extracted with either 45% aqueous phenol or 10% trichloroacetic acid at 4 C and, after further purification steps designed to remove lipids and degrade high-molecular-weight polynucleotides, the crude teichoic acids were fractionated on gel columns of various porosities (295). The cold-phenol-extracted product was eluted from 6% agarose columns with a K_d of 0.1 and was excluded from gels of lower porosity (Fig. 6a). Trichloroacetic acid-extracted material, on the other hand, yielded two fractions of

teichoic acid from columns of Sephadex G75 at K_d = 0.0 and 0.50; the smaller, excluded fraction was eluted from 6% agarose with a K_d of 0.15 (Fig. 6b). Treatment of the phenol-extracted teichoic acid with trichloroacetic acid in the cold gave an elution profile from 6% agarose identical to that obtained for trichloroacetic acid-extracted teichoic acid.

Subsequent analyses and chromatographic studies of chemical degradation products indicated that the phenol-extracted product was a complex of teichoic acid with lipid and protein, the lipid association being indicated by describing the complex as lipoteichoic acid (295). Trichloroacetic acid-extraction of cell contents or treatment of the complex with trichloroacetic acid resulted in disruption of the complex and the production of lipid- and protein-free teichoic acid together with smaller amounts of partially degraded lipoteichoic acid. It was suggested (295) that the apparent high molecular weight of lipoteichoic acid, as shown by its chromatographic elution properties and sedimentation value of 9.5s (1% wt/vol), might be explained by micelle formation. Negative staining of preparations of lipoteichoic acid with sodium phosphotungstate has shown uniform oval bodies 7 nm in diameter (A. J. Wicken and K. W. Knox, unpublished data). Similar micellar formations have been reported for extracted lipopolysaccharides from gram-negative organisms (239). It was also pointed out (295) that the existence of membrane teichoic acid as a complex with lipid consistent with its proposed membrane location. Lipoteichoic acid is thus regarded as native membrane teichoic acid, whereas the lower-molecular-weight lipid-free product obtained by acid extraction is degraded teichoic acid.

Lipoteichoic acids with similar properties have been extracted by the cold-phenol procedure from the cell contents of a variety of lactobacilli, streptococci, and bacilli (160, 163, 164, 296; A. J. Wicken and K. W. Knox, unpublished data) and also from *S. faecalis*, where the lipoteichoic acid is the group D antigen (279). In retrospect it is of interest to note that earlier work on cross-reactive and erythrocyte-sensitizing antigens obtained from a number of gram-positive organisms by aqueous phenol, water, or hot saline extraction can now be explained in terms of lipoteichoic acid as the reactive antigen in such extracts (see Glycero Teichoic Acids as Heterophile Antigens). Although it appears likely that all membrane teichoic acids exist as lipoteichoic acids, part of the membrane teichoic acid of *Lactobacillus casei* NCTC 6375 is extracted as lipid-free

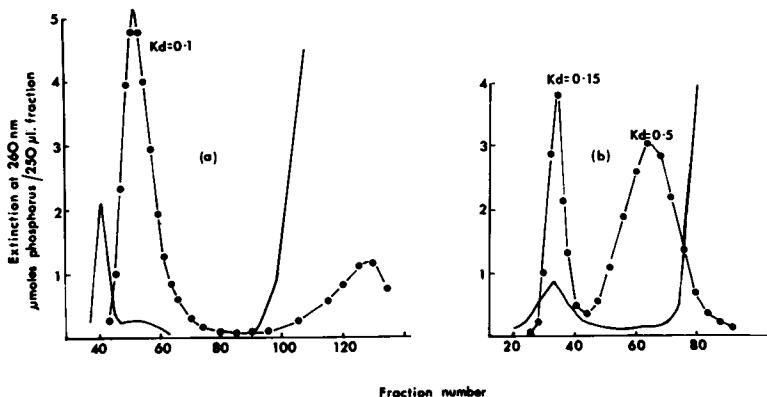


FIG. 6. Agarose gel (6%) chromatography of teichoic acid obtained by (a) phenol extraction and (b) trichloroacetic acid extraction of the cell contents of *L. fermenti*. Columns were eluted with 0.2 M-ammonium acetate, pH 6.9; ●, μmol of phosphorus per 0.25-ml fraction; continuous line, extinction at 260 nm. Fractions are approximately 4 ml K_d (distribution coefficient) data from A. J. Wicken and K. W. Knox [295].

low-molecular-weight teichoic acid by the cold-phenol procedure, and all of the membrane teichoic acid extracted by the same procedure from a strain of *Lactobacillus fermentum* appears to be of low molecular weight (A. J. Wicken and K. W. Knox, unpublished data); these variations may reflect a higher lability of the linkage of the lipid moiety to the teichoic acid in these organisms.

In a comparative study of other methods of extracting lipoteichoic acid from whole organisms of *L. fermenti* NCTC 6991, instead of the subcellular fractions, it has been shown that some of the procedures commonly used for extracting lipopolysaccharide from gram-negative organisms are applicable to the extraction of lipoteichoic acid (294). The most frequently used method for obtaining lipopolysaccharide, low in protein, involves extraction with hot, aqueous phenol (288), and this method proved to be the most efficient for extracting lipoteichoic acid from *L. fermenti*. Crude extracts were heavily contaminated with high-molecular-weight polynucleotides, but treatment of extracts with deoxyribonuclease and ribonuclease at pH 7, followed by chromatography on 6% agarose, resulted in considerable purification, and the isolated lipoteichoic acid was lower in polynucleotide content and associated protein than that obtained by the cold-phenol procedure. A lower protein content for hot-phenol-extracted lipopolysaccharide in comparison with the cold-phenol-extracted product has also been reported (287), and it has been proposed that under the conditions of hot-phenol extraction a protein-lipid-polysaccharide complex is degraded to a lipoprotein and a lipopolysaccharide (303). There appeared to be no

difference between cold- and hot-phenol-extracted lipoteichoic acids in terms of apparent molecular size of the micelles and sugar substitution of the teichoic acid, although, as might be expected, a greater loss of D-alanyl esters was noted with extraction at the higher temperature.

Lipoteichoic acid complexes, containing high amounts of associated protein, have been obtained from whole organisms of *L. fermenti*, and other lactobacilli by a mild procedure involving partial removal of cell lipids from freeze-dried organisms with chloroform-methanol (2:1, vol/vol) at 20°C, followed by aqueous extraction of the lipoteichoic acid-protein complex with water at various temperatures (294, 296). Chloroform-methanol extraction would produce a lipid-depleted membrane although no change was detected in the normal membrane profile by electron microscopy (294). In the subsequent extraction of cells with water, the lipoteichoic acid-protein complex was removed, with the concurrent disappearance of the membrane profile in electron micrographs, whereas the cell wall still appeared intact. Sufficient damage can also be imparted to the membrane by freeze-drying to allow for a partial release of lipoteichoic acid by aqueous extraction, and a similar partial release of the complex from *L. fermenti* following "cold shock" has also been observed (294). The high protein content of these aqueous-extracted lipoteichoic acids could be considerably reduced by hot-phenol extraction or digestion with papain followed by cold-phenol extraction and gel chromatography on 6% agarose (294).

The choice of method for extracting lipoteichoic acid relates to both specific requirements

and the organism concerned. Extraction from whole organisms is attractive from the point of view of simplicity of procedure. The chloroform-methanol-water-extracted product, having a high associated protein content, is a good immunogen (294), but may be variably contaminated with cell wall polymers. Hot, aqueous phenol extraction of whole organisms yields a lipoteichoic acid which is low in protein and a poor immunogen but, at least in the case of *L. fermenti*, free from contamination with cell wall material. Where the presence of wall material in lipoteichoic acid extracts is likely to interfere with subsequent operations, such as would be the case with organisms possessing a wall teichoic acid, extraction of lipoteichoic acid from subcellular fractions would be the method of choice. Here cold-phenol extraction should yield a good immunogen, whereas hot-phenol extraction yields a product low in associated protein.

It should be noted that lipoteichoic acids bind very tenaciously to ion-exchange celluloses or gels and cannot be eluted from them without partial degradation through deacylation (294). Further purification of extracts of whole organisms, although still preserving the hydrophobic nature of the lipid moiety of lipoteichoic acids, may therefore demand the application of such techniques as preparative electrophoresis or immunoabsorption. In this latter connection, concanavalin A and similar glycosyl-specific lectins may prove to be as useful in the purification of lipoteichoic acids as they have been in the case of wall teichoic acid (74).

Nature of the lipid-teichoic acid association. Lipoteichoic acid preparations from *L. fermenti* contained glycolipid indistinguishable from the free glycolipid of the cell membrane and small amounts of phospholipid (295). Lipoteichoic acids extracted in our laboratories from other lactobacilli similarly contain glycolipid, but the association with phospholipid is variable and when present may represent a mixed micelle of lipoteichoic acid and phospholipid (A. J. Wicken and K. W. Knox, unpublished data).

The linkage of glycolipid with teichoic acid in *L. fermenti* lipoteichoic acid appears to be covalent in that the glycolipid is not removed by organic solvents or hot phenol. Deacylation of the polymer removes fatty acid residues and destroys the micellar nature of the original complex, but the disaccharide glycerol portion of the glycolipid is still attached to the teichoic acid (295). Deacylated lipoteichoic acid contains no detectable phosphomonoester groups, and the glycolipid glycoside is completely re-

leased, free of esterified phosphate, during alkaline hydrolysis of the polymer. These results are consistent with a linkage of the terminal phosphate group of the teichoic acid with a sugar hydroxyl group of the glycolipid glycoside. Periodate oxidation studies showed that the glycerol portion of the glycolipid was not involved in a phosphate linkage and that the minimum length of the polyolphosphate chain was 29 glycerophosphate residues (A. J. Wicken and K. W. Knox, unpublished data). More recently (279), the lipoteichoic acid from *S. faecalis* has been shown to be a 28-unit, kojibiosyl-substituted, glycerophosphate polymer linked to membrane glycolipid by a presumed phosphodiester bond similar to that proposed for *L. fermenti*. In neither case, however, has the linkage actually been proved. In both lipoteichoic acids the presumed phosphodiester bond is more labile to the action of trichloroacetic acid than the interpolymer phosphodiester bonds, and the glycolipid glycoside and fatty acid esters are lost from trichloroacetic-acid-extracted teichoic acid.

Phosphatidylglycolipids (glycerophospholipids; 261), in which a phosphatidyl residue is attached to a sugar hydroxyl group of a glycolipid, have been reported as membrane components of a number of gram-positive bacteria (6, 71, 86, 260, 261). In at least one case, *S. faecalis*, there is a remarkable constancy of disaccharide substitution (kojibiose) of glycolipid, phosphatidylglycolipid, and lipoteichoic acid (279). In *L. fermenti*, galactosyl-1,2-glucosyl substitution of both glycolipid and lipoteichoic acid has been shown (295; A. J. Wicken and K. W. Knox, unpublished data), and in *S. aureus*, gentiobiose is found as the disaccharide substituent of both membrane teichoic acid and glycolipid (223, 260). Evidence for the glycolipid moiety of *S. faecalis* lipoteichoic acid being a phosphatidylglycolipid has been afforded by a study of the products of hydrofluoric acid hydrolysis of the polymer (279), but the linkages of the teichoic acid and the phosphatidyl group to the glycolipid are not known. It is suggested (279) that these involve different sugar hydroxyl groups (Fig. 7a). Alternatively, it is possible that the phosphatidyl group, bearing one instead of two fatty acid residues, is also the terminal glycerophosphate unit of the teichoic acid portion of the lipoteichoic acid (Fig. 7b). If this latter possibility proved to be true then these three classes of membrane lipid components, glycolipid, phosphatidylglycolipid (acylated glycerophosphorylglycolipid), and lipoteichoic acid (acylated polyglycerophosphorylglycolipid) form a graded series.

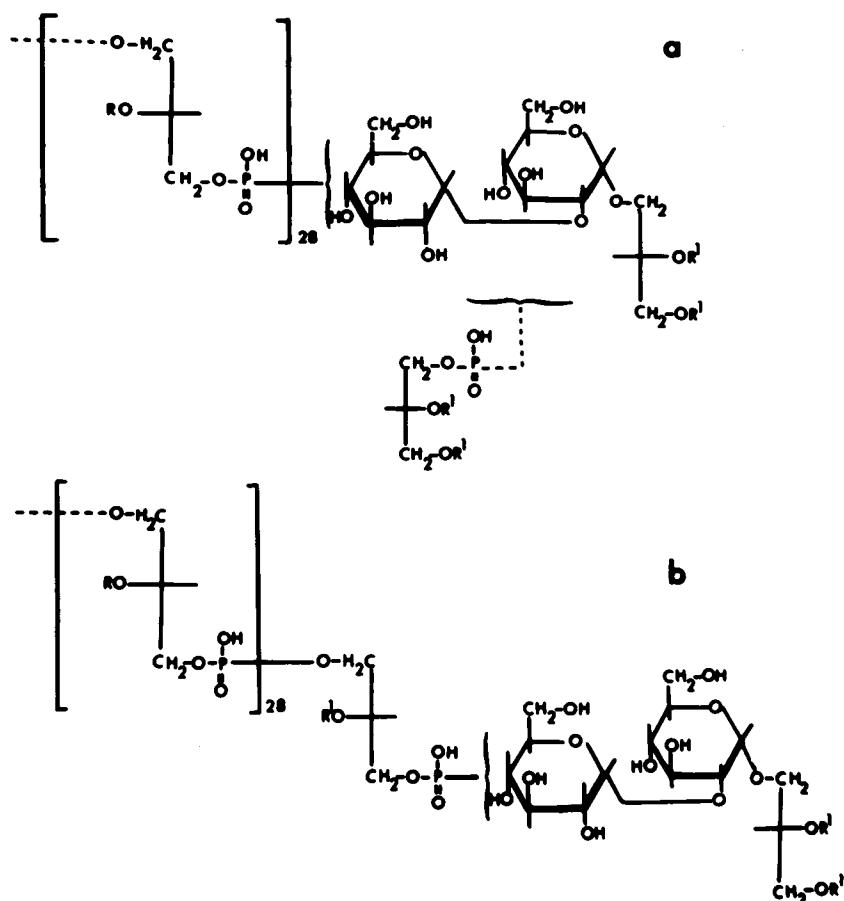


FIG. 7. Postulated nature of the lipid-teichoic acid association in *S. faecalis* lipoteichoic acid showing (a) the phosphatidyl group as a separate substituent of the glycolipid to the teichoic acid chain (279) and (b) an alternative structure in which the phosphatidyl group, bearing a single fatty acid residue, forms part of the main teichoic acid chain. R, glycosyl or H; R', fatty acid ester residue.

Isolated lipoteichoic acids are also complexed with protein, the amount varying with the extraction procedure and influencing the relative immunogenicity of the preparation (see below). The possibility of the protein being of membrane origin and complexed with the glycolipid component is suggested by studies with *Mycoplasma pneumoniae*. *M. pneumoniae* glycolipids, similar in structure to those present in the membrane of gram-positive bacteria (220, 221, 229), are not immunogenic but can be rendered immunogenic by aggregating with membrane proteins from *Archelasma* (formerly *Mycoplasma*) *laidlawii*; interaction was achieved by dispersing the glycolipids and lipid-depleted *A. laidlawii* membrane in sodium dodecyl sulphate followed by dialysis against buffer containing magnesium ions (230).

Criteria of Purity of Teichoic Acid Complexes

It is evident that with both wall and membrane teichoic acids, newer methods of isolation have aimed at obtaining these polymers in a covalent association with other cellular components. In such circumstances, the usual criteria for chemical purity are not relevant, for we are dealing with either "units" of the cell wall derived by random enzymic digestion or of the cell membrane obtained by disorganizing the membrane structure and therefore containing lipid and protein. That it is these complexes, rather than "pure" teichoic acids, which are much more likely to display immunological and other biological properties is the subject of much of the remainder of this review.

Although "purity," in the sense of a single molecular species, cannot be applied to such complexes, it is essential that their components be defined both qualitatively and quantitatively. It is regrettable that, in many instances, crude extracts of teichoic acids used in serological or other biological investigations have received no further chemical characterization than the chromatographic demonstration of polyols and polyol phosphates in acid hydrolysates. Furthermore, the assumption of glycosidic substitution of a teichoic acid by the finding of reducing sugars in acid hydrolysates is rarely valid, for contamination of teichoic acid complexes by polysaccharides is a fairly common occurrence. Here, more rigid chemical definition of glycosidic union of sugar with polyol is required, which generally involves isolation and characterization of glycosides derived from alkaline, enzymatic, or hydrofluoric acid hydrolysates of the polymer or complex (for reviews of methods see references 10, 13, 14, 305).

Definition of a teichoic acid complex in terms of a single chromatographic peak or a single precipitin line in immunodiffusion or immuno-electrophoresis could be regarded as a first step in establishing the "purity" of a complex. However, at the risk of stating the obvious, it must be remembered that, on the one hand, gel chromatography alone establishes only a degree of homogeneity of molecular size as opposed to kind and, on the other hand, a single precipitin line merely marks the presence of a single, reactive antigenic species in what might be a complex mixture. Analysis of immune precipitates and the use of fractions derived from partial degradations of complexes as inhibitors in serological reactions will often yield useful information as to the reactive components of these complexes.

Teichoic Acids as Surface Components

Reaction of antibodies with intact cells has usually been taken to imply a surface location for the antigenic components or rather, "by placing an antigen on the cell surface, we are implying that it behaves as though it were there. By placing it beneath the surface we mean that it seems . . . to be overshadowed by some other bacterial component" (301).

With the subsequent identification of specific wall components, a surface location frequently became synonymous with wall, so that we find reference to "the unexpected absence of group D antigen from the cell walls of group D streptococci" (270), and membrane teichoic acids of

streptococci and lactobacilli are termed "intra-mural cementing substance" (181, 272).

Wall teichoic acids. The spatial organization of teichoic acid-peptidoglycan complexes within the cell wall has received relatively scant attention. In *B. megaterium* strain M (204), the walls are composed of approximately 50% peptidoglycan and 50% teichoic acid; hot formamide extraction of the teichoic acid reduced the apparent thickness of the walls by 50%, leaving the rigid peptidoglycan layer, and suggesting that the teichoic acid was located mainly as a plastic layer on the outer cell wall surface. Extraction of the teichoic acid from *L. arabinosus* cell walls, on the other hand, produced no obvious change in their electron microscopic appearance (11). Studies on *B. licheniformis* (136) showed a considerable heterogeneity of the molecular organization of the three wall polymers of this organism, peptidoglycan, teichoic acid, and teichuronic acid. Some 50% of the peptidoglycan was free of linkage to the other two polymers, whereas substituted glycan strands contained either teichoic acid or teichuronic acid substituents. These findings are in keeping with the ideas of attachment of teichoic acid molecules to newly synthesized glycan strands before cross-linking of the latter into the cell wall (190) and close association of biosynthesis of wall polymers through a common membrane polyisoprenol phosphate carrier (8). Mauck and Glaser (190) interpret their findings of essentially random insertion of new cell wall polymers in *B. subtilis* by suggesting a tangential arrangement of peptidoglycan strands, rather than their being layers parallel to the cell surface, newly synthesized glycan strands with attached teichoic acid molecules being intercalated with old ones. Such a model would provide for an overall exposure of at least part of the total teichoic acid at or near the cell surface.

The uptake of antibody to teichoic acid by whole cells and isolated cell walls has been used as a probe for the location of teichoic acid within the wall (43). Cell walls of *B. subtilis* 3610 bound only 19% of the total possible antibody, but the uptake was markedly increased by a brief exposure to lysozyme; this was interpreted as "a loosening of the peptidoglycan network to allow access of antibodies to teichoic acid in a deeper layer of the wall or a previously sterically unfavored situation." That whole cells of this organism bound only 10% less antibody than purified cell walls was suggested to indicate that little or no teichoic acid was located on the inner surface of the cell wall; it is

unfortunate for unequivocal interpretation that this particular strain appears to contain identical wall and membrane teichoic acids (43). Similar results were claimed for *B. subtilis* W23, where the wall and membrane teichoic acids are different, but no experimental details were given (43). In contrast, *B. licheniformis* walls bound 71% of the calculated amount of antibody to teichoic acid, with only a slight increase on lysozyme treatment, suggesting a "surface" location of the antigen (43). The marked difference in the results with *B. licheniformis* and *B. subtilis* may reflect the differences in the structure of the two teichoic acids, with the former containing glycosyl residues as part of the main polymer chain and the latter being a classical glycosylated polyolphosphate. The precise configuration of the highly charged teichoic acid molecules in the cell wall is largely dependent on the electrostatic and hydrogen bonds formed with other wall components (144, 183, 184), and the effect with *B. subtilis* may be at least partly due to an alteration in surface charge through adsorption of the basic protein, lysozyme.

The affinity of concanavalin A for α -D-glucosylated teichoic acids has been used as a marker for the teichoic acid in the walls of *B. subtilis* 168 (Birdsell, Doyle, and Morgenstern, personal communication). Electron micrographs of this organism showed a discontinuous layer on the surface of whole cells and the outer surface of cell walls after treatment with concanavalin A. The layer was absent from cells not treated with lectin or washed with methyl- α -D-glucoside solutions to remove concanavalin A. A mutant strain that does not produce a wall teichoic acid was unaffected by treatment with concanavalin A. There also appeared to be an accumulation of the presumed teichoic acid-concanavalin A complex at the site of septum formation. These authors suggest that the teichoic acid in this organism is situated at the outer surface of the cell wall and that the individual molecules are oriented perpendicularly to the long axis of the cell because of their negative charge and highly hydrophilic nature; such an orientation would be in keeping with the Mauck and Glaser (190) model of tangential intercalation of newly synthesized teichoic acid-peptidoglycan into the cell wall.

Thus the question of the precise location of wall teichoic acids in or on the peptidoglycan network is still an open one. The possibility that teichoic acid conformation rather than depth of location within the wall is a more important factor in determining accessibility to antibodies

as well as reaction with them needs much closer examination.

Membrane teichoic acids. A membrane location for lipoteichoic acids might suggest that they would not be able to react with antibodies *in situ*. Yet in certain instances, antibodies specific for the membrane teichoic acid will agglutinate whole organisms, the best known example probably being the group D streptococci (257). Antibody reaction with the cell surface can also be shown by the removal of specific antibodies from serum, and this procedure provided evidence for *L. fermenti* membrane teichoic acid being a surface component (128, 160).

In view of the generally recognized structure of the gram-positive bacterial cell, a reaction of whole organisms with antibodies specific for a membrane component requires that antibodies either penetrate the cell wall to react with the membrane or react with membrane components protruding through the cell wall or, alternatively, that both phenomena are occurring. In support of the first proposal, Burger's suggestion (43) that the peptidoglycan architecture of the wall be considered as a highly porous network or as a sheet with large-size discontinuities has received some support from electron micrographs of freeze-etched cells of *S. lactis* (140). These studies showed the existence of "pores" in the wall, the calculated size being theoretically sufficient for the passage of molecules up to 3×10^6 daltons, i.e., an immunoglobulin M (IgM) molecule should be able to enter the pores. However, detailed studies of the passive permeability of the cell wall of *B. megaterium* (245) showed a much lower threshold of polymer exclusion in the range of 3 to 5×10^4 daltons. If this lower limit proved general among gram-positive bacteria, then even immunoglobulin G (IgG) molecules would be unlikely to penetrate the cell wall.

The absorption of membrane lipoteichoic acid-specific antibodies by whole organisms of *L. fermenti* and *L. casei* has been examined in more detail (283). These two organisms differed markedly in their behavior towards standard serological tests. *L. fermenti* absorbed and was readily agglutinated by anti-lipoteichoic acid antiserum, and the IgM fraction was more effective than the IgG fraction. *L. casei*, on the other hand, neither appeared to absorb nor was agglutinated by such antisera. A more sensitive method of detecting adsorbed antibody, involving electron microscopy of organisms that had been treated successively with antiserum and ferritin conjugated to goat anti-rabbit- γ -

globulin, showed the apparent difference between the two organisms to be quantitative rather than absolute (283). *L. casei* showed, by this technique, some surface adsorption of teichoic acid antibody, but it was irregular in distribution and significantly less than the confluent labeling shown by *L. fermenti*. A wall-membrane model has been proposed to explain these differences in surface reactivity of a membrane antigen (283). The model (Fig. 8) is based on current hypotheses on membrane structure (228, 267). The lipid bilayer depicted is asymmetric with glycolipid derivatives present only in the outer half of the bilayer. This is admittedly speculative, although there is evidence for membranes displaying such asymmetry with respect to lipid and carbohydrate components (39). Lipoteichoic acid molecules are shown as being held either in or on the membrane by hydrophobic interaction of their glycolipid moieties with membrane lipid or protein; the role of magnesium ions (283) in maintaining the integrity of lipoteichoic acid association with the membrane suggests also that ionic bonds may be involved. The long, polar polyolphosphate chains are proposed to extend by intercalation into the peptidoglycan-polysaccharide net-work of the cell wall and may come near enough to the outer surface of the cell wall for part of the polyolphosphate chain to act as a surface antigen. Factors on which this latter condition might be dependent include (i) thickness of the cell wall and degree of peptidoglycan cross-linking, (ii) the length of the polyolphosphate chain of the lipoteichoic acid, and (iii) the conformation of the chain within the ionic environment of the cell wall. The walls of *L. fermenti* and *L. casei* are quite different in their composition and lysosyme sensitivity (157, 161), and it is suggested (283) that these different milieux may allow for greater penetration of the wall matrix by lipoteichoic acid in *L. fermenti* than in *L. casei*; localized differences in wall composition may also explain the "patchy" nature of exposure of *L. casei* lipoteichoic acid at the surface as evidenced by the pattern of ferritin labeling.

This wall-membrane model suggests a highly orientated arrangement of the polyglycerophosphate chains of the lipoteichoic acid molecules. Enzymatic introduction of D-alanine ester residues into *L. casei* membrane teichoic acid (234) requires a ligase that is only effective with teichoic acid bound to membrane in, as the authors suggest, a highly orientated state; the ligase was totally ineffective with membrane-free teichoic acid. The model also suggests that

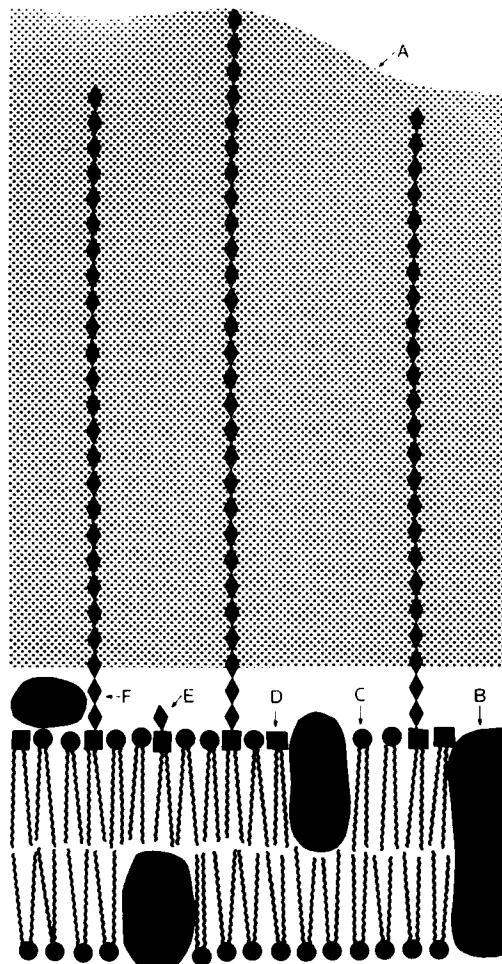


FIG. 8. Diagrammatic representation of the cell wall (A) and membrane of a gram-positive organism. The membrane components shown are protein (B), phospholipid (C), glycolipid (D), phosphatidyl glycolipid (E), and lipoteichoic acid (F). Depending on the length and conformation of the glycerophosphate chains and the thickness of the wall, the lipoteichoic acid molecules may function as surface antigens; from *Journal of Ultrastructure Research* (283).

lipoteichoic acid forms a physical link between wall and membrane and is held by hydrophobic and possibly ionic forces in the membrane and by ionic interactions between teichoic acid and peptidoglycan in the wall.

Extracellular Teichoic Acid?

In several instances, serological studies on antigens known to be teichoic acids employed the culture fluid as a source of material. Such products may have derived from viable orga-

nisms or alternatively from autolysis of dead organisms. Pollock (222) has defined the term "extracellular" as referring to products that have "originated from the cell without any alteration to cell structure greater than the maximum compatible with the cell's normal processes of growth and reproduction." An early example of a wall teichoic acid being detected in the growth medium is the soluble polysaccharide A of *S. aureus* (298), which has been identified as a teichoic acid-peptidoglycan complex (108). It might be assumed that such complexes derive from autolysis of dead organisms. However it has been demonstrated (189) in *B. subtilis* W23 and *B. megaterium* KM that the cell wall shows extensive turnover during the exponential phase of growth. Peptidoglycan and teichoic acid showed identical turnover rates resulting in almost 50% loss of cell wall material per generation in *B. subtilis* and about 30% in the case of *B. megaterium*. The extent and importance of wall turnover to wall synthesis and growth in gram-positive bacteria generally remains to be defined. The products of the wall turnover in *B. subtilis* (189) were found in the growth medium as peptidoglycan-teichoic acid complexes and could, according to the above definition, be regarded as extracellular products. Because it is likely that the same hydrolytic enzymes are involved in both wall turnover and autolysis of dead organisms, the distinction between extracellular or autolytic products, as applied to teichoic acid-peptidoglycan complexes in the growth medium, becomes largely philosophical and, in a practical sense, probably relates mainly to the phase of growth of the organism.

There are also a number of instances where reactive antigens present in growth media, and detected by their ability to adsorb to red blood cells, have the properties of membrane lipoteichoic acids. The earliest examples were streptococcal (90, 117, 156, 215) and staphylococcal culture filtrates (90, 215), whereas one study showed that the amount present in a donor bottle of blood contaminated with a *Bacillus* species was sufficient to render the recipients' red-blood cells "polyagglutinable" (50) because of the presence in human sera of antibodies reacting with the sensitizing antigen (51, 52).

Other investigations concerned with common or heterophile antigens (see later section) showed that serologically reactive material could be detected in the medium from 18- to 24-h cultures of *Listeria monocytogenes* (205) and various species of the genera *Streptococcus* (272, 274) and *Bacillus* (9). A more detailed study on a variety of gram-positive bacteria

showed that "large amounts" were detected after 24 h of incubation, with a decrease occurring after 4 days, and no activity being demonstrable after 9 days at 37°C (225). The description of "large amounts" was not given a quantitative basis, but depended on the dilution of culture fluid capable of sensitizing erythrocytes, which were then agglutinated by specific antisera; hemagglutination, detectable by macroscopic examination, was also the procedure employed by the other investigators. The method, which requires that the membrane teichoic acid be present as a lipid complex (see below), is quite sensitive, for 2.5 µg of purified lipoteichoic acid per ml is sufficient to sensitize erythrocytes and cause visible agglutination on the addition of specific antiserum (128). As membrane lipoteichoic acid probably constitutes 1 to 2% of bacterial cell mass, it can be seen that even a concentration of 2.5 µg/ml in the culture fluid would represent a substantial proportion of the cellular teichoic acid.

Whether these lipoteichoic acids should be regarded as extracellular products or as released by autolysis of dead organisms has not been resolved. Turnover of membrane components in gram-positive bacteria has not been widely investigated. In *B. subtilis*, membrane lipid showed turnover at approximately the same rate as cell wall (193), but in *B. megaterium* membrane lipid was largely conserved during cell growth and division (195). In gram-negative bacteria, the outer membrane lipopolysaccharide may be released into the growth medium (162, 240), and it has been shown (214) that *Salmonella typhimurium* lipopolysaccharide is synthesized in the plasma membrane before being translocated through the peptidoglycan layer into the outer membrane. By analogy, it is possible that lipoteichoic acid in gram-positive bacteria could lose its association with the membrane and be translocated completely through the cell wall into the external medium. The release of lipoteichoic acid, by whatever means, could also be an important factor in influencing its immunobiological properties (see later section).

CONSTANCY OF OCCURRENCE AND STRUCTURE OF TEICHOIC ACIDS

Wall Teichoic Acids

Effects of growth conditions. Studies with a variety of bacilli have indicated that the cell wall is one of the most dynamic and phenotypically variable structures of the whole cell, with large shifts in the chemical composition in response to relatively small changes in the

environment being well documented. Such changes in structure are of obvious importance in serological classification of bacteria, where it is required that antibodies be formed against a "surface" component and then react with that component.

Marked changes in the wall composition of several bacilli as a phenotypic response to changes in the growth environment have been shown in an extensive series of chemostat studies (79, 80, 82, 83, 278). *M. lysodeikticus* (*M. luteus*; 32, 33), normally considered not to produce a wall teichoic acid, can be induced to do so when chemostat-grown under conditions of limiting magnesium (83). Under conditions of limiting magnesium, potassium, nitrogen, or sulphate walls of several strains of *B. subtilis*, *B. licheniformis*, *B. megaterium*, and *S. aureus* H contained teichoic acid, but under conditions of phosphate limitation teichoic acid was replaced by teichuronic acid or a similar acidic polysaccharide (83). With *B. subtilis* var. *niger*, transition from fully magnesium-limited to fully phosphate-limited growth, or vice versa, involved a more rapid transition of polymer type than could be explained by dilution with newly synthesized wall material, and it was concluded that the control of synthesis of both polymer types was the expression of a single genotype. In *B. subtilis* var. *niger* increasing growth rate increased the proportion of teichoic acid in the cell wall; the synthesis of teichoic acid could be maintained under conditions of phosphate limitation by applying a constraint to magnesium uptake by having high concentrations of sodium ions in the external environment. Lowering the pH also increased the wall teichoic acid content in this organism as well as the extent of D-alanyl ester substitution of the polymer. Differences in the extent and nature of the glycosidic substitution of teichoic acids were also noted under different conditions of growth; Boylen and Ensign (37) also reported that the glucose content of the teichoic acid from *B. subtilis* differed with the age of the cells. The extensive turnover of the cell wall demanded by these changes has been demonstrated by labeling experiments with *B. subtilis* W23 and *B. megaterium* (189), as previously discussed.

The biological detection of such changes in wall composition is apparently limited to the observation that *B. subtilis* W23 develops phage receptor sites when grown under conditions where teichoic acid, rather than teichuronic acid, is synthesized (24). It has been suggested, however, that the inability to obtain grouping antisera reacting with strains of *L. acidophilus* is related to the variability in composition of the

cell wall, the substantial amounts of neutral polysaccharide present in walls of exponentially growing cells being replaced by anionic polysaccharides in the stationary phase (58).

Effects of mutation. Serological studies on mutants of enterobacteria have provided considerable evidence on the structure of their antigens and the specificity of antibodies (175, 176). Mutants of gram-positive bacteria have not been exploited to the same extent, although their potential role in gaining insight into the physiological function of wall teichoic acids has been realized (306). Mutants of *S. aureus* were examined for their ability to agglutinate with antibodies specific for the wall ribitol teichoic acid component, lack of reactivity indicating the possibility of (i) a change in teichoic acid structure, (ii) a lack of teichoic acid, or (iii) the presence of another surface polymer that prevented teichoic acid from reacting with the specific antibody (306); the mutant that was described fell into the third category, as the wall teichoic acid was masked by a teichuronic acid that prevented both antibody and phage adsorption. Other studies with mutants of *S. aureus*, *B. subtilis*, and *L. plantarum* have provided examples of the presence of teichoic acids lacking sugar substituents (48, 73, 97, 260), the formation of a different teichoic acid (304), the absence of teichoic acid (57, 260), and its replacement by protein (284).

Investigations on mutants of *S. aureus* indicated that the fundamental changes in composition and polymer type were associated with other phenotypic differences suggestive of surface structure changes and raised interesting questions as to the relatedness of the mutants to other taxonomic subgroups of staphylococci (304).

Variations in glycosidic substitution. Although considerable changes in wall teichoic acids may occur through mutation or growth of organisms under limiting conditions, the degree and constancy of glycosyl substitution of teichoic acids in organisms grown under normal conditions is of considerable importance to the serology of these polymers. This consideration applies, in the main, to teichoic acids of the classical type where sugars are borne as side chains on the main polyolphosphate backbone (Table 1). Biosynthesis of wall teichoic acids appears to require prior formation of the polyolphosphate, with the involvement of polyisoprenol phosphate, followed by the introduction of sugars via their uridine 5'-diphosphate derivatives (8, 14, 72, 116, 141). Baddiley and co-workers picture the enzymes for wall synthesis existing in the membrane in an ordered and

highly integrated manner as "wall synthesizing units," each unit being a multienzyme complex for the complete formation of a wall polymer (8). The units are proposed to be situated on the inside of the membrane with a limited amount of shared polyisoprenol phosphate, and each unit completes a round of polymer synthesis before releasing the carrier intermediate with the growing polymer chains extruding through the membrane into the wall where they are finally located. This concept of a vectorial process of formation of wall polymers on the outside of the membrane from nucleotide intermediates on the inside is an attractive one, and the presence in the membrane of incompletely synthesized wall polymers might explain the often-observed contamination of membrane teichoic acid preparations with wall teichoic acid.

Evidence that introduction of the sugars proceeds independently of formation of the polyolphosphate is provided by the regularity with which mutants are isolated that contain unsubstituted polyolphosphate chains. Also some bacteria have wall teichoic acids which are mixtures of fully glycosylated and glycosyl-free polymers (45, 49, 96, 135), and in *S. aureus* strains there is serological evidence that the α - and β -N-acetylglucosaminyl substituents of the wall teichoic acid occur on different polymer chains (200, 280). There are also a number of examples indicating that sugar substitution is both partial and random (14, 21), a situation rather unusual in the synthesis of structural polymers.

Membrane Teichoic Acids

Although wall teichoic acids display a variety of structures, and both the amounts present and their structures are susceptible to change, membrane teichoic acids display a much narrower structural diversity and are always of the classical, polyglycerophosphate type. Even under conditions of phosphate starvation, which leads to the replacement of wall teichoic acid by teichuronic acid, membrane teichoic acid is still synthesized (83).

A report on the effect of growth conditions on membrane teichoic acid production (191) suggested that teichoic acid synthesis, measured serologically as the group D antigen, was dependent on the glucose concentration and final pH of the medium, and good yields were only obtained from some strains of group D streptococci when 0.5 to 1.0% glucose was used in unbuffered media (final pH 4.0 to 4.2). However, because the group D antisera used to detect the teichoic acid are glucosyl specific,

these results may reflect the influence of growth conditions on the extent of glucosyl substitution of the teichoic acid rather than the extent of synthesis of the polyglycerophosphate backbone of the polymer, the latter being the important part of the molecule in its role of maintaining a high concentration of bivalent cations in the region of the membrane (126). That variations in the extent of sugar substitution of membrane teichoic acids may occur as a response to growth conditions is of general significance to serological classification and warrants further investigation.

L-forms of group D streptococci (129, 270) do not produce detectable membrane teichoic acid when they are grown in a liquid medium containing penicillin. The possibility that penicillin was inhibiting synthesis was suggested by the observation that a stable L-form derived from a group D streptococcus did produce the group-specific teichoic acid when grown in a glucose medium in the absence of penicillin (270); the teichoic acid was present in the medium, a not unexpected location because of the known release of membrane teichoic acid into the medium during protoplast formation (123, 263).

Magnesium ions are known to be of importance for the integrity of protoplast membranes (35, 87, 237, 297), and it has been shown that protoplasts of *L. fermenti* retain lipoteichoic acid in the presence of magnesium ions, but that the polymer is readily lost from protoplasts prepared in their absence (283). It has been proposed that membrane lipoteichoic acids function as ion exchangers, particularly in regard to magnesium ions (126), which are required in the synthesis of peptidoglycan and teichoic acids (8, 134). The mechanism of biosynthesis of membrane lipoteichoic acids has not received the same attention as has been given to the wall components; polyisoprenol phosphate does participate in the synthesis of a polyglycerophosphate in *B. licheniformis* (8), but it is not known whether the final location of this product is wall or membrane.

The continued formation of membrane teichoic acid under conditions where synthesis of wall teichoic acid ceases (83) might indicate a different route of synthesis. Earlier suggestions (70) that glycolipids might be involved as saccharide carriers in the synthesis of various wall polymers in bacteria had been discounted on the grounds of the lack of demonstration of metabolic turnover of glycolipid (259). However, the association of glycolipid with membrane lipoteichoic acid reopens the question of a role for glycolipid in the synthesis of these

polymers as an acceptor for growing or completed teichoic acid chains. In this respect it may be relevant that L-forms derived from *Streptococcus pyogenes* (55) and *S. aureus* (286) contain twice as much free glycolipid in their membranes as the protoplasts derived from these organisms.

IMMUNOGENICITY OF TEICHOIC ACIDS

To determine whether a bacterial component is immunogenic requires suitable detecting systems and, as discussed in the next section, the methods most used with teichoic acids are precipitation and agglutination, the latter employing bacterial cells, cell walls, or erythrocytes sensitized with the antigen. However, the various classes of antibodies differ in their capacities to elicit different serological results; in general IgG antibodies are more effective precipitins than IgM antibodies, whereas IgM antibodies are the more effective in agglutination reactions (219). Immunoglobulin A antibodies generally display both properties (219). Thus, conclusions on the relative antibody response to different immunogens can be influenced by the method of detection. The classes of antibodies produced against teichoic acids have been determined in only a few instances, and in each case the precipitin method was used (Table 2).

Wall Teichoic Acids

The immunogenicity of wall teichoic acids has been amply illustrated by the formation of specific antibodies on injection into rabbits of whole organisms representing a diverse range of gram-positive bacteria, examples including the ribitol teichoic acids of *S. aureus* (119, 243, 280), *B. subtilis* (28) and *L. plantarum* (164, 254), and the glycerol teichoic acids of *S. epidermidis*

(*albus*) (1), *B. licheniformis* (139), and group E lactobacilli (254). Grov and Rude (110) failed to obtain antibodies to teichoic acid on injection of *S. aureus* wall preparations, and a similar result with *S. epidermidis* led to the conclusion that "structures present in cell walls have been altered or lost during preparation of the walls" (109). In neither of these cases did the procedure for wall preparation include the addition of proteolytic enzymes, but loss of protein from the wall could account for the observations, e.g., trypsinizing the wall of *L. plantarum* NCIB 7220 results in the loss of immunogenicity of the ribitol teichoic acid component (164). Crude cell wall preparations of *L. plantarum* contain contaminating immunogenic membrane teichoic acid, and the most effective means of removing the contaminant while retaining the immunogenicity of the wall component involved treatment with hot, sodium dodecyl sulphate (164).

Acid-extracted teichoic acids are not immunogenic in rabbits (43, 118), although immunogenicity may be regained by forming complexes with appropriate particulate acceptors such as methylated bovine serum albumin, cetyl pyridinium chloride (43), or chromium chloride-treated erythrocytes (40). The lack of antibody response to acid-extracted teichoic acid probably is related to its low molecular weight. Immunogenicity is related to molecular weight, and the antibody response of rabbits to both dextran (149) and type III pneumococcal polysaccharide (133), for example, decreased as the molecular weight decreased from $>10^5$ to $\leq 10^4$.

The problem of obtaining antibodies to *S. aureus* ribitol teichoic acids by injecting cell fractions has suggested the use of alternative means of detection of the antigen. Both the *Helix pomatia* A hemagglutinin (113) and concanavalin A (233) react with α -N-acetyl-

TABLE 2. Classes of antibodies reacting with teichoic acids in precipitin tests

Organism	Prep injected	Antibody source	Teichoic acid source	Predominant antibody class			References
				IgM	IgG	IgA	
<i>S. aureus</i>	Organisms	Rabbit serum	Wall	+			307
<i>L. plantarum</i>	Organisms	Rabbit serum	Wall	+	(+)		164
<i>S. aureus</i> ^a	Organisms	Human serum	Wall		+		186
<i>L. plantarum</i> ^b	Lipoteichoic acid	Rabbit serum	Membrane	+	(+)		164
<i>S. aureus</i>	Organisms	Guinea pig milk	Wall			+	180
<i>S. aureus</i>	Organisms	Human nasal secretions	Wall			+	63

^a Presumed infective organism.

^b Lipoteichoic acids from *L. casei* and *L. fermenti* give similar results when examined by the hemagglutination reaction (283).

glucosaminyl residues of ribitol teichoic acid, although there is the disadvantage that they will also react with other α -D-glycosyl substituents (100, 114).

Most human sera also contain antibodies reacting with *S. aureus* teichoic acids (280), and increases in antibody content occur in patients with infected burns (268) and with staphylococcal infections in general (62, 185). The detection of antibodies by double diffusion in agar has proved to be a satisfactory method of distinguishing patients with staphylococcal endocarditis from those with endocarditis caused by other organisms (60).

The injection into humans of acid-extracted teichoic acid from *S. aureus* Copenhagen gave an increase in the amount of precipitating antibody (280). This result, which contrasts with that obtained by injecting ribitol teichoic acid into rabbits (118), may reflect differences in the immune responses of humans and rabbits; dextran, for instance, is immunogenic for man, but not for rabbit (150). However, a strict comparison of these results may not be valid for it involves comparing a (presumed) secondary response in humans with a primary response in rabbits.

Studies on the occurrence of human antibodies reacting with *S. aureus* teichoic acid have used preparations from strains in which β -N-acetyl-glucosaminyl determinants predominate. However, this determinant occurs in a number of other antigens, and cross-reactions have been shown between such teichoic acids and group A streptococci (147), group L streptococci (153), and the ubiquitous peptidoglycan (108). Without other evidence it is, therefore, difficult to decide whether the antibodies reacting with the *S. aureus* teichoic acid do, in fact, represent antibodies produced, either wholly or partly, as a result of infection by *S. aureus*. The need to qualify conclusions was realized in a recent study showing that the reaction of human sera with a variety of organic dusts could relate in part to the presence of β -N-acetylglucosaminyl-substituted teichoic acid or of "a substance with antigenic determinants related to teichoic acid" (84).

Membrane Teichoic Acids

Although studies on the immunogenicity of wall teichoic acids have usually started with the observation that injection of whole organisms will yield specific antibodies, this method will frequently fail to yield antibodies to the membrane teichoic acid. This lack of reactivity probably is related to its location rather than to an inherent lack of immunogenicity. Thus Shattock (257) discussed the "notorious difficulty"

of obtaining antibodies to strains of group D *Streptococcus bovis* and, in one of the first applications of the Mickle shaker, showed that injection of disrupted bacteria yielded potent group D antiserum. Similarly, the injection of intact *L. plantarum* organisms into rabbits only occasionally yields antibodies to the membrane teichoic acid, whereas all rabbits injected with crude cell wall produced antibodies to the contaminating membrane teichoic acid (164). Injection of whole organisms of *L. casei* yields antibodies to the wall polysaccharide (the group antigen), but not to the membrane teichoic acid, whereas injection of *L. fermenti* results in the formation of antibodies to the membrane teichoic acid (the group antigen) and not to the wall polysaccharide (157, 160, 254). The various factors that may influence the degree of penetration of lipoteichoic acid molecules into the cell wall (see section on teichoic acids as surface components) may similarly influence the immunogenicity of the membrane components when whole organisms are injected.

The influence of cellular location on immunogenicity has also been observed in studies on the formation of antibodies to peptidoglycan and cell wall carbohydrate polymers. In a discussion of results with group A and A variant streptococci, Schleifer and Krause (246) noted that antisera to group A variant organisms are a good source of antibodies to peptidoglycan and that whole cells will absorb the antibodies, whereas group A streptococcal antiserum contains little or no antibodies to peptidoglycan, and the organisms do not absorb antipeptidoglycan antibodies. The peptidoglycan of *B. licheniformis* also is only weakly antigenic, and antibodies mainly are formed against the wall teichoic acid component (139); removal of the teichoic acid greatly enhances the immune response to the peptidoglycan.

These results on the immunogenicity of peptidoglycan might also provide examples of antigenic competition (139), a description applied to the observation that simultaneous immunization of an animal with two or more antigens might result in a diminution of immune response to one or more of these as compared with control animals receiving a single antigen. Examples have been known for 70 years; recently, possible mechanisms were discussed and evidence was presented that the competition is at the level of antigen "processing" or "localizing" (41). An understanding of the mechanism of antigenic competition might also provide an explanation of the observed differences in the immunogenicity of the *L. casei* and *L. fermenti* wall and membrane components.

Depending on the method of extraction, "pu-

“rified” membrane teichoic acids may be obtained that retain their immunogenicity. Burger (43) showed that injection of phenol-extracted material complexed with methylated bovine serum albumin or cetyltrimethyl ammonium bromide proved to be an effective means of obtaining antibodies, with trichloroacetic acid-extracted material being much less effective; injection of teichoic acid preparations (unspecified) with Freund complete adjuvant failed to result in antibody formation. Subsequent studies (160) with *L. fermenti* membrane teichoic acid, with Freund complete adjuvant and the Burger injection procedure, showed that the phenol-extracted material was immunogenic, the high-molecular-weight trichloroacetic acid-extracted fraction was less effective, and that the low-molecular-weight trichloroacetic acid-extracted fraction was not immunogenic.

The extraction of membrane teichoic acid with trichloroacetic acid is an extension of its use for obtaining soluble teichoic acid from wall, where there is the requirement to hydrolyze the covalent linkage between teichoic acid and peptidoglycan. However, as discussed earlier, membrane components may be solubilized by milder procedures that yield lipoteichoic acid-protein complexes (294). The relationship of protein content to immunogenicity of *L. fermenti* products injected in Freund complete adjuvant is shown in Fig. 9. Preparation 1 was obtained by cold, aqueous phenol extraction of the soluble fraction from disintegrated organisms (160, 295) and preparation 2a was obtained by aqueous extraction of chloroform-methanol-water extracted organisms (294). Deacylation of preparation 2a and recovery of the teichoic acid fraction gave a product with a reduced protein content and also reduced immunogenicity; the antibody content of these sera was measured with preparation 2a because deacylation, by disaggregating the complex, decreases its ability to precipitate antibody (160, 163). Products with lower protein content and a corresponding decrease in immunogenicity were obtained by hot, aqueous phenol extraction (preparation 2c) and by digestion with papain followed by cold, phenol extraction (preparation 2d). Attempts to achieve a more complete removal of protein by other chemical and enzymatic methods, including digestion with trypsin and pepsin, were unsuccessful. The failure of trypsin to remove all of the protein associated with membrane lipoteichoic acid is relevant to observations that its use to obtain “pure” cell wall (61) may give preparations still containing membrane components, as indicated by lipid analyses (194) and detection of lipoteichoic acid (164).

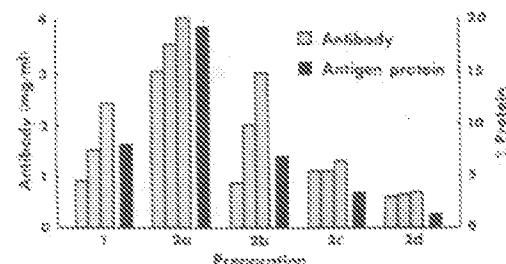


FIG. 9. Comparison of protein content of lipoteichoic acid preparations from *L. fermenti* and their immunogenicity upon injection into rabbits. Preparation 1 was obtained from disintegrated organisms by extraction with cold, aqueous phenol and preparation 2a was obtained from whole organisms by extraction with chloroform-methanol followed by hot water; preparations, 2b, 2c, and 2d were obtained from 2a by treatment with dilute alkali, hot aqueous phenol, and papain, respectively. The amount of antibody produced by each of the three rabbits injected with each preparation was determined by the quantitative precipitin method. [Data from A. J. Wicken, J. W. Gibbens, and K. W. Knox (294).]

Freund complete adjuvant is necessary for effective antibody production against lipoteichoic acids, and replacement by incomplete adjuvant results in a lessened response (160). Freund complete adjuvant consists of *Mycobacterium tuberculosis* in an oil. The original suggested (91) that the oil component of the injected emulsion serves to slow the release of the immunogen from the water droplets and also to provide protection from rapid degradation in the tissues, has been supported by recent studies (309). White (289) discussed the specific effect of the mycobacterial component of adjuvant on the immune response and concluded that it depends on the surface-active properties of the peptidoglycolipid component of wax D, possibly by increasing the capture or persistence of the immunogen at sites on the dendritic cell surface or, to use his own descriptive phrase, the peptidoglycolipid may “form the bird-lime which holds the lymphocytes more effectively in the net of dendritic cells.” The peptidoglycolipid, which consists of peptidoglycan linked to a mycolate residue of an arabinogalactan, is considered to be a product of autolysis of the cell wall (103), and recently a water-soluble adjuvant-active fraction has been obtained from mycobacterial cell wall by lysozyme digestion (3).

DETECTION OF THE ANTIGEN-ANTIBODY REACTION

Reactions of Cells and Cell Walls

Evidence for antibodies reacting with the cell surface may be obtained by showing either the

reaction of antibodies with the cell or the removal of antibodies from serum. Detection of adsorbed antibodies, generally shown by the agglutination reaction, may also employ labeled antibody; colonies of group D streptococci react with fluorescein-labeled antibodies to their group-specific membrane teichoic acid (68), and *L. fermenti* cells react with ferritin-labeled antibodies, the ferritin being detected on the cell surface (283).

The reaction of cell wall teichoic acids with antibodies would be influenced by the previously discussed conformation of the teichoic acid chains and their accessibility. An example of the influence of accessibility of teichoic acid to antibody on serological reactivity is shown by *S. aureus*, for agglutination of cells seems to depend primarily on a protein component (protein A) and type-specific products, and the teichoic acid probably is not an agglutinogen (132, 209). However, wall preparations contain very little, if any, of the type-specific products (209), and the agglutination of such preparations from several strains has been shown to depend on the teichoic acid component, specifically on the linkage between the N -acetyl-glucosamine residues and ribitol (200, 209). Wall from *S. aureus* strain Copenhagen contains both α - and β -*N*-acetylglucosaminyl-substituted teichoic acids, but antibodies could only be detected to the α substituent (209). The wall was agglutinated by heterologous antibodies specific for β -*N*-acetyl-glucosamine, so that the results would indicate lack of formation of antibodies to the β substituent on injection of strain Copenhagen rather than the unavailability of the groupings for reaction with antibody.

The agglutination reaction has been successfully applied in particular cases to the differentiation of bacterial strains, such as the typing of group D streptococci (255). However, attempts to classify lactobacilli by their agglutination reactions have not given encouraging results (252).

Precipitin Reaction

A more satisfactory method for the serological classification of lactobacilli was developed by Sharpe (251) and was based on the formation of a precipitate between antiserum and an acid extract of the bacterial cells (170). Most of the strains could be allocated to one of six groups with teichoic acids subsequently being shown to be the grouping antigens for four of these (254). The qualitative precipitin method is very suitable for screening a large number of strains, but it depends on a subjective assessment, and a

negative reaction does not necessarily mean absence of the component (257).

Of necessity, the precipitin method requires a solution of the cell fraction, but "solution" is now extended to include sonically treated wall preparations such as those from *B. licheniformis* (139). Where the solution is represented by the cytoplasmic fraction of cells, cross-reactions may occur which minimize the application of the procedure; for instance, Pease (218) showed cross-reactions between group D streptococci, *Haemophilus* species, and *Mycoplasma hominis*.

Quantitation of the precipitin reaction is achieved by analysis of the antigen-antibody precipitate for its protein content (150), generally after dissolution in dilute alkali. By adding increasing amounts of antigen to a constant amount of antiserum, the amount required for maximum antibody precipitation (the equivalence zone) can be determined (150). The precipitate can also be analyzed for the teichoic acid component to show whether the preparations contain molecules of teichoic acid differing in their determinants. This technique provides evidence of *S. aureus* teichoic acid chains having either α or β substituents (200, 280), *B. subtilis* 3610 wall teichoic acid having substituted and unsubstituted chains (96), and *L. plantarum* NCIB 7220 wall teichoic acid having chains with different degrees of glucosyl substitution (164).

The results with *L. plantarum* also indicated that only 30% of the acid-extracted teichoic acid was precipitated by antibody, which suggests that many of the chains were of too low a molecular weight to precipitate antibody. The relation between the amount of antibody precipitated and the molecular weight of the antigen has been well established by studies on dextran (102) and has been confirmed with several preparations of teichoic acid. Low-molecular-weight trichloroacetic acid-extracted teichoic acid from *L. fermenti* precipitated only 30% of the amount of antibody precipitated by high-molecular-weight lipoteichoic acid; after deacylation with consequent loss of hydrophobic interaction between teichoic acid molecules, the latter product behaved like the low-molecular-weight fraction in the precipitin reaction (160). Deacylation of phenol-extracted lipoteichoic acid from *Lactobacillus helveticus* (163) and *L. plantarum* (164) decreased by 50% the amount of antibody precipitated.

Performing precipitin tests in agar by the double diffusion (Ouchterlony) method enables the detection of a mixture of antigenic components and of showing whether different bacteria

contain a particular antigen. This procedure distinguishes between the *S. aureus* teichoic acids carrying α -and β -*N*-acetylglucosaminyl substituents and enabled the presence of one or both in a particular strain to be established (66, 132).

The ionic properties of teichoic acids enable the application of electrophoresis and immunoelectrophoresis. Teichoic acids separated by electrophoresis at pH 7 in agarose can be visualized by staining with Toluidine Blue (R. Mollenhauer, B. Sc. Hons. thesis, University of New South Wales, Sydney, 1968). The application of immunoelectrophoresis to extracts of group A streptococci showed that in addition to polyglycerophosphate, a slower-moving component (E4) was present, and the fusing of the lines indicated a serological relationship (300). Subsequent work (178) showed that component E4 contained D-alanine residues, thus accounting for its lower mobility, whereas the cross-reaction depended on the common glycerol phosphate backbone. Immunoelectrophoresis has also been used to separate teichoic acid, lipoteichoic acid, and lipoteichoic acid-protein complexes from lactobacilli and to show by appropriate absorptions that only antibodies to the teichoic acid component can be detected (294).

Hemagglutination

A method more sensitive than the precipitin reaction is hemagglutination where erythrocytes sensitized with the antigen are agglutinated by antibodies specific for the antigen. Erythrocytes from a number of animal species, including humans and sheep, are suitable (36), although in some instances adsorption is increased by treating the erythrocytes with trypsin or tannic acid (127). Studies with staphylococcal ribitol teichoic acids showed that they would not sensitize erythrocytes (64, 107, 132, 209) unless the erythrocytes were pretreated with chromium chloride (40). Staphylococcal "polysaccharide A," which contains ribitol teichoic acid, will sensitize erythrocytes (107), but only the peptidoglycan component unsubstituted with teichoic acid is adsorbed; the teichoic acid and teichoic acid-peptidoglycan fractions are ineffective (107, 108). Agglutination apparently depends on antibodies reacting with β -*N*-acetylglucosaminyl residues of peptidoglycan, and the reaction can be inhibited by *S. aureus* ribitol teichoic acid carrying this substituent (108). Latex beads have been used as an alternative to erythrocytes, but results with staphylococcal preparations would indicate that in this case also peptido-

glycan, and not teichoic acid, is adsorbed (64). Studies on the sensitization of erythrocytes by membrane teichoic acids from streptococci led to the conclusion that alanine ester residues were essential for loss of alanine under mild alkaline conditions resulted in loss of activity (143, 198). However, the conditions used would also have removed fatty acids, and later studies with *L. fermenti* membrane lipoteichoic acid indicated that fatty acids rather than alanine residues are required for erythrocyte sensitization (128). It was shown that alanine-free lipoteichoic acid sensitized erythrocytes, whereas a low-molecular-weight trichloroacetic acid-extracted teichoic acid, which contained alanine but no fatty acids, was inactive; treatment of a preparation of lipoteichoic acid with aqueous ammonia liberated fatty acids and markedly decreased the ability to sensitize erythrocytes.

Adsorption of lipoteichoic acid to erythrocytes is presumably analogous to the adsorption of lipopolysaccharides to erythrocytes and other cell membranes, where the lipid component (lipid A) has been shown to play an essential role, probably by forming hydrophobic bonds with membrane lipids (53, 115). The fatty acid content of lipoteichoic acid is approximately 4 to 5%, considerably less than that for lipopolysaccharides (175, 277), but it is comparable to that found to be effective in rendering polysaccharides capable of adsorbing to erythrocytes. Häammerling and Westphal (115) first demonstrated this effect when they showed that the addition of 5% O-stearoyl groups to a number of polysaccharides gave optimal erythrocyte-sensitizing properties. Additional evidence in support of the essential role of fatty acids for erythrocyte sensitization has been obtained by Slade and co-workers who showed that the hemagglutination procedure can be used for detecting antibodies reacting with esterified cell wall polysaccharides from streptococci of groups A and E (216, 269) and esterified glycerol teichoic acid from group A streptococci (188).

Effect of Ionic Concentration

The concentration of various salts is known to influence the antigen-antibody reaction, presumably because of effects on the surface charge and therefore the molecular shape of the reactants (150). Probably the first study relevant to teichoic acids concerned the specificity of antibodies to the type VI pneumococcal polysaccharide, which contains a repeating sequence of a trisaccharide joined to ribitol phosphate (232). The phosphorylated repeating unit was four to five times more potent as an inhibitor than was

the nonphosphorylated repeating unit, and a number of sugar phosphates were also effective inhibitors. It was correctly presumed that the results were being influenced by the ionization of the phosphate group for increasing the NaCl concentration from 0.9% to 11%, to minimize the charge on the phosphate groups, showed that the phosphorylated and nonphosphorylated repeating units were equally effective as inhibitors. It was also shown that the amount of antibody precipitated in 11% NaCl was only 66 to 77% of that precipitated in 0.9% NaCl (232).

Morse (196), who investigated the specificity of *Staphylococcus epidermidis* glycerol teichoic acid, obtained evidence for nonspecific inhibition of the precipitin reaction by sugar phosphates, but in other studies that suggested a role for galactose phosphate in the specificity of group N streptococcal glycerol teichoic acid (78), for ribitol phosphate as a determinant in *B. subtilis* W23 teichoic acid (49), and for mannose phosphate as a determinant of yeast phosphomannan (227), the possibility of non-specific inhibition was not examined.

More recent studies (166) on the reaction of the glucosyl-ribitol teichoic acid from *L. plantarum* NCIB 7220 with homologous antiserum has confirmed that the serological reactivity of teichoic acid is particularly sensitive to the ionic environment. The maximum amount of antibody was precipitated in the absence of salts; 0.4 M NaCl caused 40% inhibition and inhibition by other salts followed the lyotropic series. Divalent cations were more effective than were monovalent cations, probably because they formed complexes with the phosphate groups of teichoic acid. The binding of divalent metal ions by teichoic acids was indicated by studies with bacterial cell walls (126) and has received additional support from the observation that calcium ions form an insoluble salt with cardiolipin by combining with the two phosphate groups in the glycerol-phosphoryl-glycerol-phosphoryl-glycerol portion of the molecule (224).

The hydrochlorides of glucosamine and alanine methyl esters also cause nonspecific inhibition of the precipitation of ribitol teichoic acids by antibodies (166); presumably, the effect is related to the increase in ion concentration in general, for the resultant decrease in pH was not sufficient to inhibit the reaction. Nonspecific inhibition by glucosamine hydrochloride could account, at least in part, for its inhibitory effect on the precipitation of group A streptococcal glycerol teichoic acid (187) and group F streptococcal antigen by their specific antisera (299),

whereas the inhibition by the alanine ester hydrochlorides is a factor to be considered in defining the role of D-alanine as an antigenic determinant of teichoic acids (see below).

The reaction of concanavalin A with wall teichoic acids from *S. aureus* (233) and *B. subtilis* (74) is also inhibited by high salt concentrations, which explains why the precipitin reaction with some α -D-glucosylated teichoic acids in gel diffusion plates was not apparent until the agar had been washed with water to remove salt (22). Doyle and Birdsall (74) demonstrated significant lowering of the intrinsic viscosity of teichoic acid solutions when the ionic strength was raised, and they suggested that this indicated that the molecule had changed from a rigid rod conformation in a low ionic concentration to a random coil, which masks serologically reactive glucosyl groups, at high ionic concentrations. It is interesting that the inhibitory effect of salts in gel diffusion plates was only found with polymers having less than one glucosyl residue per polyol phosphate unit (22), whereas more highly substituted teichoic acids still reacted; the bulky substituents of these reactive molecules might hinder the salt-induced formation of a random coil. The inhibitory effect of salts was not observed when cell walls containing the teichoic acids were agglutinated by concanavalin A, possibly because of other wall components that modify the conformational changes of the teichoic acid molecule that might otherwise occur (144).

SPECIFICITY OF ANTIBODIES

General Considerations

Reactions between antigen and antibody that are detectable by precipitation or agglutination depend on an antigen with more than one determinant combining with one of the specific sites on the bivalent IgG or multivalent IgM molecule. The isolated determinant grouping of the antigen will combine with the antibody also, but being monovalent will not precipitate antibody. Generally, the reaction of the determinant grouping with antibody has been shown by an inhibition of the antigen-antibody reaction, although more recently the technique of equilibrium dialysis replacement has been introduced and shown to give comparable results (113). Either procedure enables conclusions to be made concerning the portion of the antigen combining with antibody by comparing the results obtained with components of the antigen derived by acid or enzymic hydrolysis or by comparing the effects of other known compounds.

The studies by Kabat and co-workers on dextrans (148, 150) indicated that the size of the antibody combining site could be studied by comparing the abilities of different oligosaccharides of glucose to inhibit the precipitin reaction, whereas studies on lipopolysaccharides (175, 176) have been instrumental in defining the roles of different sugars in a polymer, and also the linkages between them, in determining specificity. The extensive studies by Heidelberger and co-workers (124) have also shown the value of cross-reactions between one polysaccharide and antibodies to another as a means of identifying specific sugar determinants and their linkages.

Inhibition studies will generally indicate that one of the carbohydrate components of a heteropolysaccharide is the most effective inhibitor of the precipitin reaction, and the term "immunodominant," suggested by Heidelberger, (176) is generally used to describe such a component. Numerous studies, particularly on lipopolysaccharides (175, 176, 265, 266) have shown that the immunodominant sugar may be located at the nonreducing end of a carbohydrate chain, a terminal determinant, or within the chain. It has been proposed (265, 266) that the latter determinants be termed "non-terminal" rather than internal, for molecular models show that they occupy a "sterically superficial position in consequence of the primary chain buckling."

In many instances, teichoic acids have terminal determinants, and specificity depends on the carbohydrate residues attached to the ribitol- or glycerol-phosphate backbone. Evidence for non-terminal carbohydrate determinants is most likely to be obtained with teichoic acids that contain such residues as an integral part of their backbones (Fig. 3), for instance, the type VI pneumococcal polysaccharide (232). *L. plantarum* C106 forms a teichoic acid of this type (23); the repeating sequence is shown in Fig. 10. Studies on the reaction of this and related teichoic acids with concanavalin A led to the conclusion that concanavalin A was reacting with the nonterminal glucose component of the repeating sequence (22). In other studies on the reaction of teichoic acids with concanavalin A (233), it had been assumed that teichoic acids having carbohydrate units as an integral part of the backbone would not give a precipitate because a reaction depended on "multireactive sites." However, the anomalous results of Archibald and Coapes (22) could be explained by the shape of this teichoic acid molecule. Simmons (265, 266) has shown that α -1 → 2 and α -1 → 3 linkages between D-sugars have a profound effect on the configuration of a polymer, so that

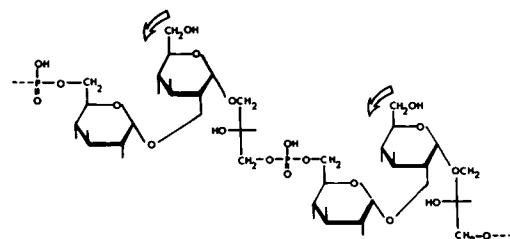


FIG. 10. Proposed effect of α -1 → 2 glycosidic linkages on the shape of *L. plantarum* C106 wall teichoic acid showing buckling of the main chain with exposure of nonterminal glucose residues that can react with concanavalin A (shown by arrows).

it may be considered as being composed of "steric repeating units," which, in the case of the lipopolysaccharides he examined, corresponded to the serological repeating units. With *L. plantarum* C106 teichoic acid, the proposed effect of the α -1 → 2 linkage on the shape is also indicated in Fig. 10; the teichoic acid does have the required multireactive sites (shown by arrows), but these are nonterminal glucose residues.

Simmons (265, 266) has also shown that the 1 → 2 or 1 → 3 linkages, which are important in defining the shape of the molecule, are not involved in the combination with the homologous antibody combining site. The term "apodeterminant" was introduced to define such determinants which "by conferring a distinctive shape in the molecule . . . determine the sequence and steric accessibility of the more distant structures, that comprise the antigenic determinant without themselves forming an integral part of it." In this context, we may consider the α -1 → 2 linkage in *L. plantarum* C106 teichoic acid as an apodeterminant, with the sterically accessible glucose residue having the required free hydroxyl groups at positions 3, 4, and 6 for interaction with concanavalin A.

Stereochemical restrictions on the shape of a teichoic acid molecule may also influence whether antibodies will be formed against a potential determinant attached to the teichoic acid backbone. Based on studies on the carbohydrate determinants of blood groups substances (172) and the Forssman antigen (264), it has been concluded that only those groups that "have a nonreducing terminal with great steric rigidity appear to be strongly antigenic and thus have greater immunogenetical significance" (264). Whether this generalization can be extended to teichoic acids will depend on whether more knowledge on the shapes of the polymers is obtained, but it might be expected that the bulky 2-acetamido grouping of the *N*-acetyl-

glucosaminyl-substituted ribitol teichoic acids of *S. aureus* and the disaccharide substituents of the *Streptococcus faecalis* glycerol teichoic acid would hamper steric flexibility; in each case these substituents are the antigenic determinants.

Because antibodies are generally formed against the substituents on the polyribitol- or polyglycerol-phosphate backbone, it has frequently been concluded that such substituents mask the backbone and thus prevent the formation of antibodies to the glycerol or ribitol components (43, 152, 178, 179). However, McCarty had noted that certain group A streptococcal sera were specific for the glycerol phosphate backbone and reacted with substituted glycerol teichoic acids. Kabat (148) discussed these results in connection with his proposition that antibodies could be formed against one "face" or surface of an antigen, and he suggested that such sera "would have a specificity complementary to the —CH— aspect of carbon 2 of the glycerol teichoic acid, and not involving the more hydrophilic side of the molecule." Other examples of antibodies specific for the glycerol phosphate sequence in reaction with teichoic acids carrying different sugar substituents have subsequently been described (163, 296), while the concept of antibodies reacting with only one face of a polymer has been well established by studies on lipopolysaccharides (175, 176, 265, 266).

Specificity of Antibodies to Carbohydrate Substituents

Teichoic acids generally have a carbohydrate substituent, and when this is the immunodominant component, the teichoic acid may prove useful in serological classification (Tables 3-5). Evidence for an immunodominant carbohydrate substituent has come from inhibition studies and also from cross-reactions. For instance, antibodies to the wall ribitol teichoic acid from *L. plantarum* NCIB 7220 cross-react with the membrane glycerol teichoic acid because of the common α -D-glucosyl substituents (164). The α -D-glucosyl-specific antibodies to both the ribitol and glycerol teichoic acids of *L. plantarum* also cross-react with dextrans although not with amylose, amylopectin, or glycogen (165). This reaction may be related to the presence of single glucosyl residues joined by 1 → 2 or 1 → 3 linkages to the main chains of dextran. The antibody specificity does not require such a linkage, but the results might provide another example of the importance of these linkages in defining the accessibility of an antigenic determinant (265, 266).

The specificity of antibodies may be such that structurally related carbohydrates can occupy the combining site. This would account for the observation that galactose inhibits the reaction between *L. plantarum* glucosyl-substituted glycerol teichoic acid and homologous antibodies and also for the reaction of these antibodies with the galactosyl-substituted glycerol teichoic acid from *L. fermenti* (164).

The extent to which antibody specificity is shared between the glycosyl substituent(s) and the backbone of polyol phosphate has been examined in only a few instances. Burger (43) provided evidence that glycerol contributed to the specificity of antibodies to the glycerol teichoic acid from *B. subtilis* 3610 by showing that 2-O- α -D-glucosyl-glycerol was a considerably more effective inhibitor than was 3-O- α -D-glucosyl-glycerol; however, glucose must be the major contributor, for the antiserum did not precipitate unsubstituted glycerol teichoic acid. A similar conclusion was reached with the glucosyl-ribitol teichoic acid of *L. plantarum* NCIB 7220, where α -D-glucosyl-ribitol was a more effective inhibitor than methyl- α -D-glucoside, but the antiserum did not react with unsubstituted ribitol teichoic acid (164).

With glycerol teichoic acids, the relative contributions of glycosidic substituents and glycerol phosphate to specificity can be examined by comparing inhibitions by the appropriate methyl glycoside and glycerol-phosphoryl-glycerol-phosphoryl-glycerol, which is obtained from cardiolipin and contains 1 → 3 linked glycerol phosphate units. By this means it was shown (164) that antibodies to the membrane teichoic acid of *L. plantarum* NCIB 7220 differed in their specificity, depending on the preparation injected—whole "cell wall" gave antibodies specific for the glucosyl substituents, trypsinized "cell wall" that still contained residual membrane gave antibodies specific for the glycerol phosphate backbone of the membrane teichoic acid, whereas antibodies to the isolated lipoteichoic acid were specific for both glucose and glycerol phosphate.

Individual rabbit variations also lead to differences in antibody specificity. With *L. fermenti* membrane teichoic acid, where the substituents are D-galactose and α -D-galactosyl-1 → 2-D-glucose, a comparison of 11 rabbit sera showed that 100 μ mol of D-galactose gave 5 to 47% inhibition (median 32%), whereas 100 μ mol of D-glucose gave 0 to 22% inhibition (median 7%); those sera with low sugar specificity were shown to be primarily specific for the glycerol phosphate backbone (160, 296). Differences in the specificity of sera could also account for

TABLE 3. Glycerol teichoic acids as grouping antigens of streptococci

Organism	Teichoic acid			References
	Location	Substituent(s) ^a	Determinant	
Group D	Membrane	Glc- α -1 → 2-Glc		291, 292
Group N	Membrane	Gal-phosphate ?	Glc- α -1 → 2-Glc ?	78, 125
<i>S. mutans</i>				
Group I (a)	Membrane	Glc, Gal	β -Gal	281, 282
Group II (b)	Wall	Gal	β -Gal	34 ^b

^a See Table 1 for abbreviations.^b A. S. Bleiweis, personal communication.

TABLE 4. Group antigens of lactobacilli

Serological group	Group antigen	Location	Components ^a	Immuno-dominant component	References
A	Teichoic acid	Wall? Membrane	Glycerol, α -Glc	α -Glc	163, 192, 254
B	Polysaccharide	Wall	Rha, Glc, Gal, GlcNAc, GalNAc	α -Rha	98, 157, 158
C	Polysaccharide	Wall	Glc, Gal, GlcNAc, GalNAc	β -Glc	98, 157, 158
D	Teichoic acid	Wall	Ribitol, α -Glc	α -Glc	159
E	Teichoic acid	Wall	Glycerol, α -Glc	?	164, 254
F	Teichoic acid	Membrane	Glycerol, Gal, Gal- α -1 → 2-Glc	α -Gal	21, 254
					160 ^b

^a Rha, L-rhamnose; see Table 1 for other abbreviations.^b A. J. Wicken and K. W. Knox, unpublished data.

TABLE 5. Teichoic acid antigens of staphylococci and micrococci

Staphylococcus	Polyol	Substituent ^a	Polysaccharide synonyms	Occurrence in micrococci	References
<i>aureus</i>					
strain Wood 46	Ribitol	β -GlcNAc	A = A β	Biotypes 2-5, 7	66, 119, 211
strain 263	Ribitol	α -GlcNAc	263 = A α	—	132, 211
<i>epidermidis</i>					
strain T1	Glycerol	α -Glc	B = B α	Biotypes 1, 6	1, 65, 66, 211, 212
strain T2	Glycerol	β -Glc	B β	—	65, 66, 211

^a See Table 1 for abbreviations.

conflicting conclusions on whether group D streptococcal teichoic acid cross-reacts with antisera to group A streptococci (78, 179).

D-Alanine as an Antigenic Determinant

In contrast to the observation that antibodies are frequently formed against the carbohydrate components of teichoic acids, there are only a few instances of antibodies with specificity for the D-alanine substituents, namely, certain rabbit antisera to group A streptococci (178, 188) and *S. aureus* phage type 187 (152). This lack of immunogenicity may be related to the marked lability of the alanine ester linkage, for D-alanine components of peptidoglycan (246) and poly-D,L-alanyl-proteins (244) have been shown to be immunodominant.

Evidence for D-alanine being an immunodominant component of teichoic acids was provided by the loss of serological reactivity concomitant with the hydrolysis of ester linkages and by D-alanine methyl ester hydrochloride being a better inhibitor of the precipitin reaction than L-alanine methyl ester hydrochloride; D-alanine, itself, did not inhibit (152, 178, 188). However, the alanine ester hydrochlorides can cause nonspecific inhibition similar to that caused by other ionized compounds (166). Treatment of *L. plantarum* NCIB 7220 ribitol teichoic acid with ammonia gave an alanine-free product retaining 95% of its serological activity with homologous antiserum, yet the precipitin reaction was inhibited 32% by 100 μ mol of D,L-alanine methyl ester hydrochloride

and 35% by 100 μmol of L-alanine methyl ester hydrochloride. Injection of *L. plantarum* cell wall, from which alanine esters were removed by hydroxylamine, gave antiserum whose reaction with homologous teichoic acid was similarly inhibited by the ester hydrochlorides; for each system 100 μmol of sodium chloride gave 33% inhibition. From these results, it may be concluded that the reported inhibition by the L-alanine methyl ester is nonspecific and that the specific effect of the D-alanine component of the ester is less than has been generally concluded.

Antibodies Specific for Glycerol Phosphate

McCarty (177) showed that antibodies specific for the glycerol phosphate backbone of teichoic acids could be obtained from certain rabbits injected with group A streptococci; evidence was provided by reaction of the sera with unsubstituted glycerol teichoic acid and by inhibition by synthetic polyglycerophosphate (average chain length = six units). Glycosylated teichoic acids from lactobacilli also cross-react with group A streptococcal antibodies, and the cross-reaction is inhibited by prior absorption of the serum with polyglycerophosphate (163). Analysis of the precipitate formed with *L. fermenti* lipoteichoic acid confirmed that glycosyl-substituted teichoic acid was being precipitated.

Additional evidence for a sequence of glycerol phosphate units being immunogenic determinants in glycosyl-substituted teichoic acids has come from studies with glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) as an inhibitor (296). In the reaction between *L. helveticus* lipoteichoic acid and homologous antiserum, the antibodies are primarily specific for the glucosyl substituents, and 2 μmol of G_3P_2 gave only 5% inhibition, possibly because of nonspecific ionic inhibition. However, for lipoteichoic acids from *L. plantarum*, *L. fermenti*, and *L. casei*, where there is a much lower degree of substitution, 2 μmol of G_3P_2 was a very effective inhibitor and varied from 35 (*L. fermenti*) to 75% (*L. casei* NCTC 6375). The size of the immunogenic determinant has not been established, although specificity does require that the glycerol units be joined by phosphodiester bonds involving positions 1 and 3 of glycerol, for the wall teichoic acid from *B. stearothermophilus*, which has phosphodiester bonds between positions 2 and 3 of glycerol (290), did not react.

The degree to which specificity of sera related to the glycerol phosphate backbone was reflected in the cross-reactions between the lac-

tobacillus teichoic acids (296) and the results for *L. helveticus*, *L. fermenti*, and *L. casei* are compared in Fig. 11. Also included are results showing the extent to which each of the reactions that precipitated sufficient antibody was inhibited by 2 μmol of G_3P_2 (296). A comparison of the two sets of results shows the importance of glycerol phosphate determinants in the cross-reactions.

The inhibition of the precipitation of glycerol teichoic acid by the glycerol phosphate component of cardiolipin suggested the use of the sera to detect cardiolipin; cardiolipin is employed in several complement fixation tests for syphilis, and antibodies to cardiolipin are specific for the phosphoryl glycerol phosphate component (112). It was shown (293) that rabbit antibodies specific for the glycerol phosphate component of lipoteichoic acids reacted as reagins in the Kolmer complement fixation test, although not in the more specific tests for treponemal infections; reagins produced in the few cases of human syphilitic infection that were tested did not react with lipoteichoic acid. These results could explain some of the biological false-positive reactions for syphilis obtained in cases where there was a recent history of gram-positive bacterial infection (93) and a consequent possibility of a high titer of antibody to teichoic acid.

That human sera may contain a significant titer of antibody to membrane teichoic acids has been shown by a number of studies. In most of these, as is discussed in the next section, the

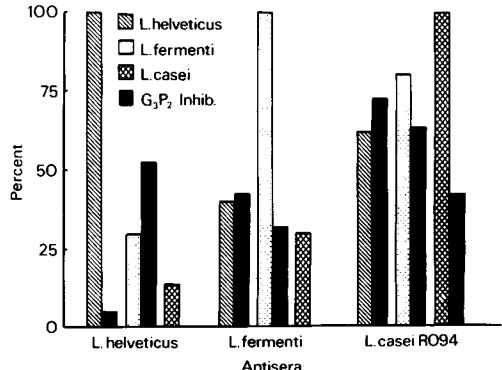


FIG. 11. Cross-reactions between lipoteichoic acids from *L. helveticus*, *L. fermenti*, and *L. casei* R094 and rabbit antisera to the lipoteichoic acid preparations. Results are expressed as a percentage of the amount of antibody precipitated in the homologous reaction. Where sufficient antibody was precipitated, the percent inhibition of the reaction by 2 μmol of glycerol-phosphoryl-glycerol-phosphoryl-glycerol is also shown. [Data from A. J. Wicken and K. W. Knox (296).]

structure of the reactive teichoic acid was not known. However, a comparison of the reactivity of 53 human sera with erythrocytes sensitized with lipoteichoic acids from *L. plantarum*, *L. fermenti*, and *L. casei* showed that 32 had detectable antibodies, and that 17 reacted with each of the teichoic acids and contained cross-reacting antibodies (182).

The presence of cross-reactive antibodies specific for the polyglycerophosphate backbone could account, at least in part, for the observations that human sera contain antibodies agglutinating erythrocytes sensitized with extracts of oral streptococci (171) and teichoic acids from two *Bacillus* species (67). In a study on oral streptococci, where a positive correlation was obtained between the amount of dental caries and antibody reacting with extracts of certain cariogenic streptococci, it was assumed that the antibodies detected were specific for the particular strain, and a causal relationship was invoked (171). In another study (67), on "naturally occurring antibodies to bacillary teichoic acids," the presence of cross-reacting antibodies was even more certain, for the authors show that specificity depends on the polyglycerophosphate backbone.

Glycerol Teichoic Acids as Heterophile Antigens

The conclusion that glycerol teichoic acids differing in glycosyl substituents can react with antibodies specific for the glycerol phosphate sequence would account for a number of observations on cross-reactions between gram-positive bacteria from diverse groups and has led to the use of such terms as nonspecies specific (225), Rantz (104), heterophile (179), or heterogenetic antigens (51).

The first definitive evidence was provided by McCarty's study (177) with the group A streptococcal antiserum that reacted with polyglycerophosphate, and a precipitate was obtained with acid extracts from a variety of gram-positive, but not gram-negative, bacteria. Antiserum to a strain of *L. acidophilus*, which reacts with acid extracts of various gram-positive bacteria (252, 253), has also been shown to contain antibodies specific for the polyglycerophosphate backbone (253).

Most studies on heterophile antigens of gram-positive bacteria have used the more sensitive hemagglutination method for detection. The reaction of human sera with erythrocytes sensitized with culture filtrates from streptococci and staphylococci (90, 215) was interpreted as indicating a common antigen, and evidence for a more wide-spread occurrence of such antigens

among gram-positive bacteria was obtained by Rantz and co-workers (225, 226); they also showed that hot-water extraction of cells gave active material, introduced phenol extraction, and obtained suitable rabbit antisera.

The identification of intracellular teichoic acid as an antigenic component (177) led to the suggestion by Salton that the erythrocyte-sensitizing antigen(s) known to be wide-spread among gram-positive bacteria might also be teichoic acid (see 104). This was shown to be the case, for McCarty's polyglycerophosphate inhibited the hemagglutination of erythrocytes sensitized with lysates of *S. aureus* (104). Confirmatory evidence was obtained by Stewart (271), who inhibited the agglutination of erythrocytes sensitized with streptococcal culture fluids containing "Hickey antigen" by adding synthetic polyglycerophosphate.

Neter and co-workers conducted a detailed study of erythrocyte-sensitizing antigens of gram-positive bacteria and improved the method of detection (206), provided evidence for placental transfer of antibodies reacting with Rantz antigen (207), and extended the list of reactive organisms to include *L. monocytogenes* (205); this observation provided a basis for the known serological cross-reaction between *Listeria* sp. and *S. aureus*. Not all gram-positive bacteria examined gave an active product, and negative results were obtained, for instance, with *Bacillus brevis* (9) and "*M. lysodeikticus*" (*M. luteus*) (104). Other investigators had also noted that not all gram-positive organisms produced a reactive component, but in many cases a negative reaction could relate to difficulties in extracting and detecting the antigen. For instance, the conclusion for *B. brevis* was based on the lack of reaction of the culture filtrate and the failure of cells to absorb antibodies (9), and a later examination of a hot saline extract of another strain did give a positive result (51). However the study on "*Micrococcus lysodeikticus*" (*M. luteus*) used lysozyme digests (104) and indicated that more research is warranted to determine whether this organism does contain the expected membrane glycerol teichoic acid.

All of the above studies indicating the occurrence of cross-reactive teichoic acids in a variety of gram-positive bacteria depended on having antibodies reacting with a common structural feature, presumably the glycerol phosphate sequence. However, antibodies to teichoic acids may also be specific for the glycosyl substituents and would account for the specificities of those human sera that differed in their ability to agglutinate erythrocytes sensitized with lipotei-

choic acids from different lactobacilli (182). Such differences in specificity would also explain the differences observed in the ability of individual human and rabbit sera to agglutinate erythrocytes sensitized with culture filtrates (272, 274) or hot saline extracts (51, 181, 273) of different gram-positive bacteria. The erythrocyte-sensitizing antigens had the properties of lipoteichoic acids, and evidence was obtained in two instances for the presence of teichoic acids (67, 271). In some cases, extracts from an individual strain reacted with sera of differing specificity, and it was assumed that this indicated that a corresponding number of antigens were present. Alternatively, the sera could be detecting different antigenic determinants on the same teichoic acid molecule.

To establish the presence in different bacteria of antigens with the same determinant grouping, investigators have generally used the classical technique of absorption of antisera with intact organisms. Where absorption occurs, there is certainly evidence for the antigen being present (225). However, conclusions based on a lack of absorption of antibodies, the main criterion used by Stewart and co-workers for delineating the antigens of streptococci and lactobacilli, do not take into account the situation that membrane teichoic acid may not be available to react with antibodies. A more satisfactory method was employed by Chorpenning and Dodd (51) who used erythrocytes sensitized with antigen for absorbing antibodies from human and rabbit sera.

TEICHOIC ACIDS AS GROUP ANTIGENS

Teichoic acids have proved to be of only limited usefulness as antigenic components in the classification of bacteria, probably because of the restricted array of sugar substituents on the polyol phosphate backbone and the variable, and apparently uncontrolled, degree of sugar substitution that can occur; disaccharide substituents which would extend the possibilities of structural diversity appear to occur only in membrane teichoic acids (Table 1). Among the streptococci, where serological identification is frequently the preferred method, teichoic acids are the group antigens in only a minority of instances (Table 3). They do, however, have a major role in the serological classification of lactobacilli (Table 4) and may also aid in the classification of staphylococci and micrococci (Table 5).

Streptococci

The reactivity of acid extracts of streptococci with specific antisera (170) has enabled approx-

imately 20 alphabetically designated serological groups to be identified. Group A streptococci, because of their pathogenicity, have probably received the most attention, and McCarty's studies defined the cell wall polysaccharide as the group antigen (179). Other studies by numerous investigators indicate that wall polysaccharides are the specific components for most of the serological groups, the principle exceptions being groups D and N (101).

The classification of streptococci into group D (Table 3) depends on a specific membrane lipoteichoic acid (43, 77, 279, 291, 292), and another division into serological types depends on specific cell wall polysaccharides (69, 75, 76, 217, 255). Because of its location, the presence of the group antigen may prove to be difficult to establish, both with regard to antibody production and the detection of the antigen in an extract (257, 270). Certain strains of streptococci originally classified into group Q on the basis of their specific cell wall polysaccharide (270) are now known to contain the group D antigen (208, 270). These strains, designated *Streptococcus avium*, differ in their physiological characteristics from the established group D enterococci, *S. faecalis* and *S. faecium*, and it has been proposed that "the group Q streptococci constitute a valid species which should be included in the serological group D streptococci" (208). Nowlan and Deibel (208) found it "difficult to reconcile" the physiological differences between the group Q and D strains "on the basis of a variation in type-specificity." However, there is no a priori reason for antigenic properties correlating with physiological characteristics, and it would seem logical to consider the group Q antigen as a type-specific polysaccharide present in certain group D streptococci.

The specificity of antibodies to the group N antigen (Table 3) has not been clarified; galactose did not inhibit the precipitin reaction, and the inhibition by galactose-1-phosphate and galactose-6-phosphate, rather than indicating that galactose phosphate is the serological determinant (78), could be another example of nonspecific inhibition by a sugar phosphate (196, 232). Further, group N antiserum cross-reacted with only one galactose-containing pneumococcal polysaccharide, type XVI, which also contained glycerol phosphate (125). Glycerol phosphate cannot be a major contributor to serological specificity, for group N antibodies did not precipitate group A streptococcal polyglycerophosphate (78).

The oral streptococci have evoked considerable attention because of their role in dental caries. Although certain of the oral streptococci

can be classified into one or other of the Lancefield groups (158), the cariogenic strains, usually classified as *Streptococcus mutans* or *S. sanguis*, do not belong to any of the known serological groups. Schemes for the serological classification of *S. mutans* have been proposed on the basis of the reactivity of cell extracts with rabbit antisera (38, 142). An examination of representative strains by Bleiweis and co-workers has shown (Table 3) that those in groups I and II (142), also called groups a and b (38), contain specific teichoic acids (34, 281, 282, A. S. Bleiweis, personal communication). There is evidence that *S. sanguis* strains also have a group-specific teichoic acid, one with the properties of a membrane component, but not extractable with trichloroacetic acid or phenol (238a; B. Rosan, Abstr. Int. Ass. Dent. Res. 1972, p. 265).

Lactobacilli

Attempts to develop a serological classification of lactobacilli have used both cell agglutination and the precipitin reaction. The results of a number of investigations on agglutinating antigens indicate a complexity of surface components, generally type-specific and with only a limited applicability to classification, for they often cut across species as defined on the basis of physiological properties (252).

Studies by Sharpe and co-workers showed that the precipitin reaction, by using acid extracts and specific antisera, provides a much more satisfactory method for the serological classification of lactobacilli. An examination of 442 strains, representing all species then recognized, showed that 70% could be classified as belonging to one of six groups and one subgroup (251) designated by the letters A to F (256); subsequently group G was defined (238). Broadly, the classification is in agreement with one based on the physiological characteristics of the strains, although strains of *L. casei* belong to one of two groups, B and C. The lactobacilli were one of the first genera to be surveyed for the occurrence of teichoic acids (31), and subsequent work has shown that they are the antigens defining four of the six groups examined (Table 4); the group G antigen has not been identified.

In group A, there have been conflicting conclusions on whether the wall or membrane teichoic acid is the group antigen, and studies using the precipitin method implicated the membrane component (254), whereas agglutination reactions suggested that the wall component was responsible (192). However, it is now apparent from studies on *L. helveticus* NCIB 8025 that the wall and membrane glycerol

teichoic acids are of similar structure, each having α -D-glucosyl substituents (163). The isolated membrane lipoteichoic acid is immunogenic, with the antibodies being primarily specific for the α -D-glucosyl substituents and, therefore, cross-reacting with wall teichoic acid (163). In terms of the reactivity of Lancefield acid extracts of group A organisms (251) or their agglutinability (192), it is to be expected that the wall teichoic acid would be the major contributor to the serological reaction because of the greater amount of teichoic acid in the wall (163). However, none of the studies on the group A lactobacilli has resolved whether the antibodies formed on injection of whole organisms are elicited by the wall teichoic acid, the membrane teichoic acid, or both. Thus the classification of a strain as group A could depend on the production of antibodies to the membrane teichoic acid and their reaction primarily with the wall component.

The group E antigen has also been identified as a glucosyl-substituted glycerol teichoic acid which is present in the wall (254); an examination of one strain, *L. buchneri* NCIB 8007, showed that there were α -D-glucosyl substituents (21). The main difference between this teichoic acid and the group A antigen is the degree of sugar substitution, and the ratio of glucose to phosphorus is 0.64:1.00 and 0.45:1.00, respectively, for the wall and membrane teichoic acids from *L. helveticus* NCIB 8025 (group A) (163) as compared with 0.26:1.00 for *Lactobacillus buchneri* (21). The *L. buchneri* teichoic acid is randomly substituted with glucosyl residues, and D-alanyl ester residues are attached to most of the remaining glycerol units (21); antibody specificity may be related to the high degree of D-alanine substitution, for specificity based entirely on α -D-glucosyl substituents would be insufficient to distinguish the group E antigen from the group A antigen.

The group F antigen is also a glycerol teichoic acid; it is present in the membrane (159, 254) and differs from the A and E antigens by containing both glucose and galactose, with galactose being primarily responsible for serological specificity (Table 4).

Group D lactobacilli, represented by most strains of *L. plantarum* (including *L. arabinosus* 17-5), contain a wall ribitol teichoic acid (15) and a membrane glycerol teichoic acid (59), each of which bears immunodominant α -D-glucosyl substituents (164); antibodies to the wall teichoic acid cross-react with the membrane component (164). The conclusion that the wall teichoic acid is the group antigen has been confirmed by observations that strains lacking

the glucosyl substituents on the ribitol teichoic acid (73, 164) or containing a different teichoic acid (4) do not react with group-specific antiserum.

In general, injection of group D lactobacilli does not yield antibodies to the membrane component, although these can be obtained by injecting crude cell wall or lipoteichoic acid in Freund adjuvant (164). A similar situation also exists with strains of *L. casei* where injection of whole organisms gives antibodies specific for the cell wall polysaccharides (Table 4), and antibodies to the membrane component can only be obtained by injecting disintegrated organisms (Knox and Wicken, unpublished data) or lipoteichoic acid in Freund adjuvant (296). The *L. casei* lipoteichoic acids contain minor amounts of glucose and galactose, although earlier studies on one of the strains (ATCC 7469 = NCTC 6375) described a trichloroacetic acid-extracted teichoic acid presumed to be devoid of carbohydrate substituents (155).

As discussed previously, antisera to a number of the purified lipoteichoic acids will cross-react with lipoteichoic acids from serologically unrelated lactobacilli because of immunodominant α -D-glucosyl substituents or glycerol phosphate units (160, 163, 296). These observations seem to militate against an efficacious serological classification of lactobacilli. However, based on the reactivity of acid extracts of organisms with antiserum to whole organisms (251), such a classification has been developed. These two parameters may well be crucial, for injection of whole organisms will frequently fail to elicit the production of antibodies to the membrane lipoteichoic acid, and acid-extracted teichoic acids react only poorly in the precipitin reaction in comparison with the high-molecular-weight lipoteichoic acids (160, 163).

Staphylococci

A serological distinction between virulent and avirulent staphylococci was first indicated by the studies of Wieghard and Julianelle (298) who isolated fractions containing serologically active phosphorylated polysaccharides from type A (virulent) and type B (avirulent) strains. The type A strains would now be classified as *S. aureus* and are generally distinguishable from type B strains by the production of coagulase and ability to ferment mannitol. The currently preferred name for type B strains is *Staphylococcus epidermidis*, although they have also been designated *S. albus* and *S. saprophyticus* (179).

The relationships of polysaccharides A and B

to teichoic acids and their current synonyms are summarized in Table 5. The rather confusing literature on the nomenclature of staphylococcal antigens also contains references to Jensen's antigen A, a component originally thought to be a polysaccharide, but now known to be a protein (54). Antigen A appears to be the major agglutinogen within *S. aureus* strains; a large number of type-specific agglutinogens are known, most of which are presumed to be proteins, whereas the wall teichoic acids do not seem to contribute to the agglutination reaction of whole organisms (209). The serological detection of teichoic acid in extracts of *S. aureus* is influenced by whether the antiserum used detects only α - or β -N-acetyl-glucosaminyl substituents, and it has therefore been concluded that tests for the detection of wall teichoic acid will not replace the coagulase test in identifying the majority of *S. aureus* strains (197).

A number of types of *S. epidermidis* can also be distinguished by cell agglutination, although the specific agglutinogens have not been identified (1). Double-diffusion precipitin reactions showed, however, that extracts from two-thirds of the strains examined contained an α -D-glucosyl-substituted glycerol teichoic acid (1). Because this precipitin method readily distinguishes between the *S. epidermidis* and *S. aureus* teichoic acids and because some strains of *S. aureus* are coagulase negative, the serological detection of teichoic acid has been proposed as a means of differentiating coagulase-negative staphylococci (1).

Micrococci

Micrococci are similar to staphylococci in their physiological characteristics and, as a result, certain strains of micrococci that have been examined for teichoic acid components have been incorrectly classified as *S. epidermidis* (173, 211) and *S. lactis* (16, 17, 32). The naming of species has also led to confusion, and it has been proposed that there should only be two, *Micrococcus roseus* and *M. luteus*, the latter including strains of *M. lysodeikticus* (32, 33).

Results of chemical (30, 109) and serological studies (174, 211) indicated that some strains contain a cell wall ribitol teichoic acid, whereas in others a cell wall glycerol teichoic acid may be present. The ribitol teichoic acid is indistinguishable, upon double diffusion in agar, from *S. aureus* β -N-acetylglucosaminyl-substituted ribitol teichoic acid (174) and has been shown (Table 5) to be present in strains representing five of the eight biotypes (211); these strains also contain an additional unidentified compo-

nent, C, which is detectable upon double diffusion in agar, and the extract containing both components is called polysaccharide A β C (174). Other strains (Table 5) contained an antigen indistinguishable from α -D-glucosyl-substituted glycerol teichoic acid upon agar gel double diffusion, whereas biotype 8 strains lacked detectable antigenic components. The results of these studies led to the conclusion that the type of teichoic acid present "should be given more weight" in the classification of micrococci (211).

IMMUNOBIOLOGICAL PROPERTIES OF TEICHOIC ACIDS

Wall-Associated Teichoic Acid-Peptidoglycan Complexes

Cell walls of various gram-positive bacteria are known to be toxic to animal tissues. Wall fragments of group A streptococci produce chronic dermal lesions in rabbits (248), injury to the joints of rabbits (249), and rheumatic cardiac-like lesions in mice (213). Dermal lesions in rabbits, similar to those produced by group A streptococci, are produced at the site of injection of cell walls of a number of gram-positive bacteria, including *S. aureus*, streptococci of groups B, C, E, F, and K, *L. casei*, *Actinomyces israelii*, and *Actinomyces (Odontomyces) viscosus* (247). Peritoneal abscess formation in mice has also been achieved with *S. aureus* cell walls (169).

The rate and pattern of degradation of different species of bacteria after phagocytosis is very variable (56), and it is known, for instance, that group A streptococcal cell walls may persist in migrating human phagocytes for sufficient periods of time to be deposited in body tissues and may thus be an important factor in the pathogenesis of post-streptococcal sequelae in humans (99). Although the toxicity of many bacterial cell walls has been established from their ability to either produce or perpetuate chronic inflammatory lesions in animal tissue, questions as to whether these are due to direct toxic effects or hypersensitivity reactions as well as the role(s) of the various antigenic components of the cell walls remain, in most instances, to be answered.

Studies on the dermal toxicity in rabbits of cell walls of several gram-positive bacteria (2, 247) have indicated clearly that the peptidoglycan component of the walls is responsible for the lesions produced. A large particle size is also important in determining the toxic activity; acid hydrolysis progressively decreases the activity. Similar conclusions concerning peptidoglycan being the toxic principle of *S. aureus* cell

walls were reached in a series of skin test studies (167) on guinea pigs with a range of carefully fractionated wall antigens. The latter included viable and heat-killed whole organisms, cell walls, peptidoglycan complexes, teichoic acid, teichoic acid-peptidoglycan complexes, and low-molecular-weight peptidoglycan fragments. In nonsensitized animals, all antigens but teichoic acid elicited acute inflammatory reactions which decreased in size after 10 h. In animals sensitized with whole organisms, the reactions to all antigens but teichoic acid and peptidoglycan fragments remained erythematous and indurated for at least 30 h and were interpreted as delayed hypersensitivity type reactions. Although teichoic acid was apparently not directly involved in the formation of these dermal lesions, it was suggested to have an indirect role; teichoic acid-peptidoglycan fragments, but not peptidoglycan fragments alone, are capable of evoking the hypersensitivity reactions in sensitized animals. This, as the authors suggest, may be due to the greater particle size of the teichoic acid-peptidoglycan fragments or their greater persistence in animal tissue through inhibition of peptidoglycan digestion by associated teichoic acid. Of the two strains of *S. aureus* used in this study, it is interesting to note that strain Copenhagen had a greater sensitizing capacity than did strain 263; the former, in common with peptidoglycan, has a predominantly β -linked N-acetylglucosaminyl-substituted teichoic acid, whereas in the latter strain the glucosamine substitution is essentially α in configuration.

Cutaneous hypersensitivity in humans to staphylococcal wall teichoic acid has been claimed (185), but the test antigen used was purified by electrophoresis from a crude extract prepared by sonic treatment of whole organisms and was likely, therefore, to have been a teichoic acid-peptidoglycan complex. Earlier reports (47, 199) that *S. aureus* cell wall teichoic acid was responsible for removing phagocytosis-promoting and killing factors for this organism in human sera have subsequently been disproved (262), and once again peptidoglycan of large molecular size appears to be the responsible cell wall component. Studies on the toxicity of surface antigens of *S. aureus*, as measured by their effects on the respiration of mouse liver tissue (154), suggested that wall-associated teichoic acid and peptidoglycan were the toxic factors involved; the various fractions and extracts tested were not, however, characterized chemically. The cellular toxicity of streptococcal peptidoglycan, noted earlier, is paralleled by a general inhibition of phagocytosis by rabbit

polymorphs, and this appears to be due to a direct toxic effect of the peptidoglycan on the phagocytic cells rather than to absorption and neutralization of opsonins and other factors (146).

Thus, on the basis of the evidence presently available, it seems unlikely that wall-associated teichoic acid has a direct role in the toxicity of cell walls.

Membrane Lipoteichoic Acids

Rheumatic fever and glomerulonephritis are well-known sequelae to β -hemolytic group A streptococcal infections, and in both cases an immunological etiology, involving humoral antibody to a streptococcal component, has been generally accepted. Controversy exists as to whether the mechanism of pathogenesis involves antibody combination with antigen fixed at the site of tissue damage, or whether combination is intravascular, involving circulating antigen followed by deposition of the immune complex at the site of subsequent tissue damage. The identity of the antigen(s) involved also has not been resolved nor whether it is truly of streptococcal origin or is instead a host tissue component capable of cross-reacting with antibody to a streptococcal antigen (241, 312).

The ready attachment of membrane lipoteichoic acids to erythrocytes by a presumed hydrophobic interaction of their glycolipid moiety with the erythrocyte membrane has been discussed earlier in this review. Sensitization of erythrocytes with streptococcal membrane teichoic acid (143, 198) has been shown to be reversible, for antigen is able to be transferred from erythrocytes to tissues and vice versa. It has been suggested (198) that this may indicate a mechanism whereby streptococcal antigen can be distributed to host tissues, and perhaps this plays a role in the pathogenesis of rheumatic fever (and glomerulonephritis). Whether or not the binding of lipoteichoic acid to animal cell membranes is nonspecific or specific in involving a finite number of "receptor-sites" remains to be determined. If the latter proves to be true lipoteichoic acid, carried by erythrocytes from the site of streptococcal infection, could become concentrated at host membrane surfaces where there are a large number of receptor sites or sites of high affinity for lipoteichoic acid. This hypothesis is attractive and, certainly, among the wide range of streptococcal antigens which have thus far been endowed with the role of causative or reactive antigen in these two diseases, lipoteichoic acid has received the least attention. A major objection to

this role for streptococcal lipoteichoic acid is its potential lack of specificity in contrast to the high degree of strain specificity of group A streptococci in causing these diseases, particularly streptococcal glomerulonephritis. However, lipoteichoic acids are invariably obtained *in vitro*, and are possibly released *in vivo*, as complexes with protein. It is possible that the associated protein provides the necessary specificity of the immune reaction, whereas the lipoteichoic acid acts as a carrier that is selective for particular host tissues.

Complement fixation by antibody reaction with erythrocytes sensitized with lipoteichoic acid and subsequent hemolysis has been demonstrated in a sheep erythrocyte-rabbit antibody system (293). Antibody agglutination of various animal erythrocytes sensitized with the Rantz antigen from a variety of bacterial sources has been referred to earlier (see Glycerol Teichoic Acids as Heterophile Antigens section), and may in the presence of complement lead to lysis. The clinical importance is well illustrated by at least one recorded case of transfusion of contaminated blood leading to severe hemolysis and death (50).

Repeated injection into rabbits of "the membrane-associated teichoic acid" from *S. pyogenes* (285) has been reported to result in marked calcification and tubular necrosis of the kidneys without any significant increase in humoral antiteichoic acid antibodies. The method of preparation of the teichoic acid was not described in this preliminary report (285), but it seems likely that the lipoteichoic acid used in earlier studies (143, 198) was involved. Removal of D-alanyl esters from the teichoic acid with alkali rendered it inactive and, as has been discussed earlier, such a procedure would also effectively deacylate the glycolipid moiety of a lipoteichoic acid.

Lipoteichoic acids are amphipathic molecules that possess both hydrophilic and hydrophobic groupings, and it would be expected that the various biological properties that have been described above, particularly those requiring adsorption to cell membranes, relate to this property. Lipopolysaccharides of gram-negative bacteria are also amphipathic molecules, and they resemble lipoteichoic acids in their physico-chemical properties. Injection of lipopolysaccharides into higher animals causes a number of characteristic and well-known reactions, including fever, shock, and death (endotoxic reactions), and they also stimulate bone resorption in tissue culture (120). Preliminary observations indicate that lipoteichoic acid is not pyrogenic for rabbits nor lethal for mice (A. J. Wicken and

K. W. Knox, unpublished data). It does, however, give a positive localized as well as a generalized Schwartzman reaction in rabbits, the latter being accompanied by the bilateral cortical necrosis of the kidneys characteristic of a lipopolysaccharide-induced reaction; higher doses of lipoteichoic acid (100 to 500 µg) than for lipopolysaccharide are required for positive reaction, and lipid-free teichoic acid is without effect (A. J. Wicken and K. W. Knox, unpublished data). Lipoteichoic acid, but not lipid-free teichoic acid, also stimulates bone resorption in tissue culture, although the amounts required are approximately 10-fold greater than for lipopolysaccharide (E. Hausmann, A. J. Wicken, and K. W. Knox, unpublished data). Lipopolysaccharide has been implicated in the resorption of bone in human periodontal disease (120), and it seems likely that lipoteichoic acid from gram-positive organisms in plaque and gingival pockets could play a similar role. The "odontopathic" potential of at least one gram-positive human isolate, resembling *Streptococcus salivarius*, has been indicated by its ability to stimulate alveolar bone loss in rats (151).

The observations that the biological properties of lipoteichoic acids are less pronounced than those of lipopolysaccharides are not surprising. Although both contain lipid, the lipid moiety (lipid A) of lipopolysaccharides is unusual, and their characteristic components are hexosamine, generally glucosamine, and a 3-hydroxy fatty acid, generally 3-D-hydroxymyristic acid (235). The lipid A moiety is responsible for endotoxicity, and it seems likely that "the presence or absence of acylated hydroxy fatty acid esters may determine the degree of endotoxic activity of lipopolysaccharides" (235).

CONCLUDING REMARKS

Teichoic acids derive their name from the Greek work for "wall," and the earliest preparations were derived from this source and were considered "pure" when they could be obtained free from other cell components. Similarly, the products we now know as membrane teichoic acids were originally isolated by the same procedures and with the same intention. We now realize that preservation of covalent association of teichoic acids with peptidoglycan or membrane glycolipid and protein is important insofar as the biological activity of these polymers is concerned. Historically, the distinction between wall and membrane teichoic acid was an operational one based on disrupted organisms, i.e., the teichoic acid was either in the cell wall or it was not. However, as has been discussed in this review, membrane teichoic acids may be quite

intimately associated with the cell wall matrix in the intact cell, and the former locational distinction is less clear-cut. Also, a subsurface location for membrane teichoic acid does not necessarily mean that it is "hidden" where *in situ* serological activity is concerned. Ionic interactions with wall components and the consequent effects of these on conformation and depth of penetration of teichoic acid into the wall may be major determining factors in the activity of the polymer as a classical, surface antigen. The known variability of wall composition as a response to environmental changes may, too, be paralleled in some organisms by variations in the extent of surface activity of membrane teichoic acid. Whether or not a clear distinction between wall and membrane teichoic acids is warranted would seem to depend on the validity of certain assumptions, viz., (i) the apparently essential requirement for a membrane teichoic acid as compared with the variable occurrence of wall teichoic acids, (ii) the membrane polymer is always a polyglycerophosphate, whereas the only requirement for a wall teichoic acid, by definition, is that it contain a polyolphosphate, and (iii) the constancy of covalent association of wall teichoic acids with peptidoglycan and of membrane teichoic acids with membrane components.

Compared with cell wall polysaccharides, teichoic acids have generally found limited application in the serological classification of gram-positive bacteria. However, the more recent studies with Staphylococci, micrococci, and oral streptococci indicate that teichoic acids can be a valuable aid to classification, provided that the strains being examined are well characterized and that there are methods for extracting serologically reactive teichoic acid and of obtaining specific antiserum. Furthermore, the diversity of structure found in wall teichoic acids has potential in the study of the effects of shape of polar antigens on antibody specificity that has been little used to date.

Finally, comparisons have been made in this review between the physical and biological properties of lipopolysaccharides of gram-negative bacteria and lipoteichoic acids of gram-positive bacteria. Possibly, the teichoic acid portion of lipoteichoic acid can be regarded as analogous in many of its properties to the heptose-phosphate-containing common core of the outer membrane lipopolysaccharide of the gram-negative organism, whereas the cell wall polysaccharides, and to a lesser extent wall teichoic acids, frequently found in gram-positive organisms provide structures of greater

serological specificity that are comparable to the O-specific side chains of lipopolysaccharides.

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